Departamento
de Química Orgánica

Glicomiméticos y Glicoligandos anfifílicos. Interacciones con enzimas, receptores y ácidos nucleicos.

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Memoria presentada por el
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para optar al grado de Doctor en Química

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El presente trabajo ha sido realizado por el Ldo. Julio Rodríguez Lavado en el Departamento de Química Orgánica de la Facultad de Química de la Universidad de Sevilla, bajo la dirección de la Dra. Carmen Ortiz Mellet, Catedrática de Química Orgánica de la Universidad de Sevilla, el Dr. José Manuel García Fernández, Profesor de Investigación del Instituto de Investigaciones Químicas (CSIC-Universidad de Sevilla) y el Dr. José Luis Jiménez Blanco, Profesor Titular de Química Orgánica de la Universidad de Sevilla.

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A

ABTS	Sal diamónica del ácido 2,2'-azinobis-(3-etilbenzotiazolina-6-sulfónico).
Ac	Acetilo
Ac_2O	Anhídrido acético
AcOEt	Acetato de etilo
Ad	Adamantano
ADN	Ácido desoxirribonucleico
Anal.	*Analysis*
aq	*Aqueous*
Ar	Argon
ATR	*Attenuated Total Reflectance*

B

9-BBN	*9-Borabicyclo[3.3.1]nonane*
BHG	*HEPES/Glucose/buffer*
BMDM	*Bone Marrow-Derived Macrophage*
Boc	*terc*-Butoxicarbonilo
bp	*base pairs*
bPEI	Polietilenimina ramificada
BSA	*Bovine Serum Albumine*
^{t}Bu	*terc*-butilo
^{t}BuOH	*terc*-butanol

C

ºC	Grados Celsius
Calcd	*Calculated*
CD	Ciclodextrina
CMC	Concentración micelar crítica
ConA	Concanavalina A
COS-7	Línea celular derivada de células de fibroblastos de riñón de mono verde africano
COSY	*Correlated Spectroscopy*
CT	Ciclotrehalana
CuAAC	*Cu(I)-catalyzed Azide-Alkyne Coupling*

D

δ	Desplazamiento químico
ΔG	Variación de la energía libre de Gibbs
ΔH	Variación de entalpía
ΔS	Variación de entropía
d	*doblet*
DAPI	4 ',6-Diamino-2-fenilindol
DC-Chol	*3β-[N-(Dimethylaminoethane)carbamoyl]cholesterol*
DCM	Diclorometano
DDT-Au	Dodecanotiol-oro
DLS	*Dynamic Light Scattering*
DMAP	4-(Dimetilamino)piridina
DMEM	Medio de Eagle modificado por Dulbecco
DMF	*N*,*N*-Dimetilformamida

DMSO	Dimetilsulfóxido
DNA	Deoxyribonucleic acid
DOPE	*Dioleoylphosphatidylethanolamine*
DOSPA	2,3-dioleyloxi-N-[2(esperminacarboxamido)etil]-N,N-dimetil-1-propanaminio trifluoroacetato
DOTAP	*N*-[1-(2,3-dioleoiloxi)propil]-*N,N,N*-trimetilamonio metil sulfato
DOTMA	*N*-[1-(2,3-dioleiloxi)propil]-*N,N,N*-trimetilamonio

E

ELLA	*Enzyme-Linked Lectin Assay*
ELISA	*Enzyme-Linked ImmunoSorbent Assay*
ESIMS	*Electrospray Ionization Mass Spectrometry*
Et	Etilo
Et_3N	Trietilamina
Et_2O	Dietil éter
EtOAc	Acetato de etilo
EtOH	Etanol

F

FCS	*Fetal calf serum*
FBS	*Fetal Bovine Serum*
FT	Transformada de Fourier

G

GD	Enfermedad de Gaucher

H

h	hora
HEK-293	Línea celular derivada de células embrionarias de riñón humano transfectadas con genes MD2-TLR4
HEK-Blue™	Línea celular derivada de células embrionarias de riñón humano
HepG2	Línea celular derivada de células de hepatoblastoma humano
HEPES	Ácido 2-[4-hidroximetil piperazina-1-il] etanosulfónico
Hex	Hexanoil
HMQC	*Heteronuclear Multiple-Quantum Coherent experiment*
HRP	*Horseradish Peroxidase*

I

IC_{50}	Concentración inhibitoria del 50%
IL	Interleucina
IR	Espectroscopía infrarroja
ITC	*Isothermal Titration Calorimetry*

K

K	Kelvin
kDa	Kilodalton

K_d	Constante de disociación
K_a	Constante de asociación

L

λ_{em}	Longitud de onda de emisión
λ_{exc}	Longitud de onda de excitación
Lac	Lactosa
LDA	Diisopropilamiduro de litio
LSD	Enfermedad de almacenamiento lisosomal
LPS	Lipopolisacárido
LTX	Lipofectamine® (mezcla 3:1 de DOSPA y DOPE).
Luc	Luciferasa

M

m	*multiplet*
M3-PALS	*Mixed Mode Measurement-Phase Analysis Light Scattering*
Man	Manosa
$(ManS)_3\beta CD$	beta-Ciclodextrina monofuncionalizada con una antena trimanosilada
MD2	*Myeloid differentiation 2*
Me	Metilo
MeCN	Acetonitrilo
MeOH	Metanol
Ms	Mesilo
MsCl	Cloruro de mesilo
MMR	*Mannose Macrophage Receptor*
MTT	Bromuro de 3-(4,5 dimetil-2-tiazoil)-2,5-difeniltetrazólico
m/z	Relación masa/carga
MW	*Microwave*

N

NaOMe	Métoxido de sodio
Naph	Naftilo
NaH	Hidruro de sodio
NMR	*Nuclear Magnetic Resonance*
N/P	Relación Nitrógeno/Fosfato
NPs	Nanopartículas

P

paCD	Ciclodextrina policatiónica anfifílica
paCT	Ciclotrehala policatiónica anfifílica
pb	pares de bases
pNPP	*p-Nitrophenylphosphate*
PBS	Tampón de fosfato salino
PBST	Tampón de fosfato salino con 0.05% v/v de Tween 20
PC	Chaperona farmacológica
PCT	Terapia de chaperona farmacológica
pDNA	Plásmido de ADN
Ph	Fenilo
PI	*Polydispersity index*
PNA	*Peanut Agglutinin*
PTSA	*p-Toluensulphonic acid*
py	Piridina

R

RE	Retículo endoplasmático
rhMMR	*Recombinan Human Mannose Macrophage Receptor*
RLA	Actividad relativa de luciferasa
RMN	Resonancia magnética nuclear
RPMI	*Roswell Park Memorial Institute (culture medium)*
rt	*room temperature*

S

6S-NAdB-NJ	*N'*-[4-(Adamant-1-ilcarboxamido)butil]iminometiliden-6-tionojirimicina
6S-NOI-NJ	*N'*-Octil-iminometiliden-6-tionojirimicina
s	*singlet*
SAR	Relación estructura-actividad
satd	*saturated*
SD	Desviación estándar
SDS	Dodecil sulfato de sodio
Si-BPA-Cu(I)	Sílica-bispiridilamina-cobre (I)
Soln	*Solution*

T

T	Temperatura
t.a.	temperatura ambiente
TAE	Tampón Tris-Acetato/EDTA
TAM	*Thermal Activity Monitor*
TBE	*Tris/Borate/EDTA*
TBAB	Bromuro de tetrabutilamonio
TEMPO	*(2,2,6,6-tetramethylpiperidin-1-yl)oxidanyl*
TFA	Ácido trifluoroacético
THF	Tetrahidrofurano
TLR4	*"Toll-like" receptor 4*
TNS	6-(*p*-toluidino)-2-naftalenosulfonato
TPP	Trifenil fosfina
Ts	Tosilo
Tr	Tritilo

U

UV Ultravioleta

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Relación de Esquemas de la Tesis

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Targeted delivery of pharmacological chaperones for Gaucher disease to macrophages by a mannosylated cyclodextrin carrier†

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Gaucher disease (GD) is a rare monogenetic disorder leading to dysfunction of acid β-glucosidase (β-glucocerebrosidase; GCase) and accumulation of glucosylceramide in lysosomes, especially in macrophages (Gaucher cells). Many of the mutations at the origin of GD do not impair the catalytic activity of GCase, but cause misfolding and subsequent degradation by the quality control system at the endoplasmic reticulum. Pharmacological chaperones (PCs) capable of restoring the correct folding and trafficking of the endogenous mutant enzyme represent promising alternatives to the currently available enzyme replacement and substrate reduction therapies (ERT and SRT, respectively), but unfavorable biodistribution and potential side-effects remain important issues. We have now designed a strategy to enhance the controlled delivery of PCs to macrophages that exploit the formation of ternary complexes between the PC, a trivalent mannosylated β-cyclodextrin (βCD) conjugate and the macrophage mannose receptor (MMR). First, PC candidates with appropriate relative avidities towards the βCD cavity and the GCase active site were selected to ensure efficient transfer of the PC cargo from the host to the GCase active site. Control experiments confirmed that the βCD carrier was selectively recognized by mannose-specific lectins and that the corresponding PC:mannosylated βCD supramolecular complex retained both the chaperoning activity, as confirmed in human GD fibroblasts, and the MMR binding ability. Finally, fluorescence microscopy techniques proved targeting and cellular uptake of the PC-loaded system in macrophages. Altogether, the results support that combined cyclodextrin encapsulation and glycotargeting may improve the efficacy of PCs for GD.

Introduction

Lysosomal storage disorders (LSDs) are a heterogeneous group of inherited diseases caused by genetic defects affecting lysosomal catabolic enzymes.[1] In many cases, the mutation at the origin of the disease gives rise to a protein that cannot fold properly and is subjected to endoplasmic reticulum associated degradation (ERAD) by the quality control system of the cell. Ironically, the mutant protein is often functional, but it cannot undergo trafficking to the Golgi apparatus for maturation and then to the lysosome, which results in the abnormal accumulation of cellular debris, mostly glycosphingolipid metabolites, in different cell types and tissues.[2] By mechanisms that are not fully understood, such accumulation leads to a range of potentially life-threatening pathologies. Incidence of each LSD individually is rare, though altogether LSDs affect 1 in 5000 to 10 000 live births in Western countries. Gaucher disease (GD), the most prevalent LSD (about 14% of the total), is associated

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† Electronic supplementary information (ESI) available: Synthesis of compounds **7** and **8**, NMR and ESI-MS spectra of newly synthesized compounds, CD-chaperone binding isotherms, and plots for the inhibition of commercial glycosidases and of the adhesion of rhMMR to yeast mannan. See DOI: 10.1039/c3ob42530d

with the dysfunction of β-glucocerebrosidase (GCase, EC no. 3.2.1.45), responsible for glucosylceramide (GlcCer) hydrolysis.[3] Over 300 mutations have been characterized in the gene encoding for GCase, which translates into a large array of disease manifestations, from visceromegaly in attenuated forms of type 1 GD to neurological affections in acute and subacute neuronopathic type 2 and 3 GD, and varied disease progression rates.[4]

The therapeutic goal towards GD, and LSDs in general, is restoring the balance between substrate influx and degradation.[5] Enzyme replacement therapy (ERT), in which patients are regularly supplemented with an exogenous recombinant GCase, is the main clinical treatment for GD nowadays.[6] Alternatively, other therapeutic approaches based on more "druggable" candidates have been investigated.[7] Substrate reduction therapy (SRT), based on glycosphingolipid biosynthesis inhibitors, has proven useful to reduce GlcCer influx.[8] Both ERT and SRT treatments address substrate accumulation but not the protein folding defects and their potential contributions to the pathophysiology of the disease.[9] More recently, specific ligands of the deficient enzyme capable of promoting correct folding at the ER have been shown to restore normal trafficking and, ultimately, lysosomal activity.[10] This so-called pharmacological chaperone therapy (PCT) constitutes a promising therapeutic paradigm for GD and many other protein folding disorders.[11] Someway counter-intuitively, competitive inhibitors of the affected enzyme, used at sub-inhibitory concentrations, can act as mutant enzyme activators through this rescuing mechanism.[12] In any case, the future development of PCT requires engineering small molecular entities featuring (i) highly selective affinity towards the mutant enzyme, not to disrupt parallel cellular metabolic machineries, (ii) favourable chaperoning *versus* inhibitory capabilities, (iii) membrane-diffusiveness to access cells and cellular compartments, and (iv) recognition abilities to selectively target storage affected tissues.

Recent studies have shown that bicyclic glucose mimics with amphiphilic character and GCase inhibitory properties behave as active site-directed pharmacological chaperones (PCs) for GD.[13] Among them, amphiphilic bicyclic nojirimycin analogues belonging to the so-called sp^2-iminosugar glycomimetic family[14] have proven to be able of rescuing the misfolded protein from ERAD and restoring trafficking to the lysosome,[15] where the catalytic activity is expressed. The efficacy of PCT for GD may benefit from strategies enhancing delivery of the chaperone to the cells that are primarily affected by glucosylceramide accumulation, namely macrophages (Gaucher cells). Site-specific delivery of recombinant glycosidases by cell membrane receptor-targeted carriers has already proven a promising strategy for improving ERT in the context of several LSDs,[16] but the suitability of such an approach for pharmacological chaperone delivery to macrophages remains unexplored. First, the binding affinity of the chaperone towards the carrier must be finely tuned to warrant efficient transfer to GCase. Second, the carrier must be equipped with a ligand allowing specific recognition by macrophages. Third, the PC-loaded macrophage-targeted carrier must promote cell internalization before premature PC release.

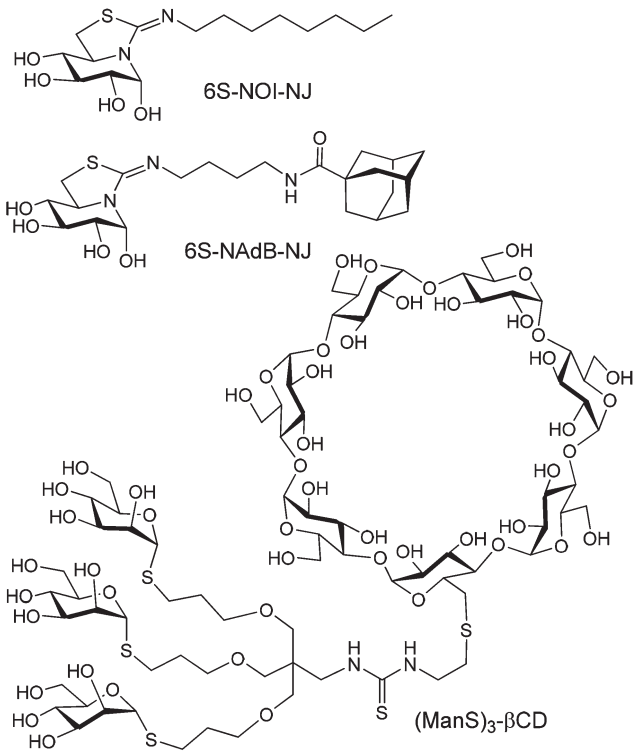


Fig. 1 Chemical structure of the sp^2-iminosugar pharmacological chaperones 6S-NOI-NJ and 6S-AdB-NJ and of the trimannosylated βCD carrier $(ManS)_3$-βCD.

Interestingly, native β-cyclodextrin (cyclomaltoheptaose; βCD) has been previously shown to facilitate transfer of amphiphilic sp^2-iminosugars to recombinant GCase in co-crystallization experiments.[17] The basket-shaped structure of CDs features a hydrophobic cavity that can accommodate guest molecules of appropriate size and improve their water solubility and bioavailability,[18] which has been broadly exploited in pharmaceutical technology.[19] The possibility to incorporate mannosyl moieties that are specifically recognized by the macrophage mannose receptor (MMR), a C-type lectin expressed at the membrane of macrophages and dendritic cells,[20] through selective chemical functionalization methods[21] further offers a good opportunity for optimization of macrophage-targeted PC:βCD complexes for enhanced PCT against GD. Herein, we have characterized the host–guest interactions of *N*′-octyl- and *N*′-[4-(adamant-1-ylcarboxamido)butyl]-iminomethylidene-6-thionojirimycin (6S-NOI-NJ and 6S-NAdB-NJ),[22] bearing octyl and adamantyl moieties, respectively, with the trivalent thiomannopyranosyl-tagged βCD derivative $(ManS)_3$-βCD (Fig. 1). We have further assessed how formation of the $(ManS)_3$-βCD:PC complexes influences both the interaction with glycosidases and the recognition by mannose-specific lectins. Our results demonstrate that (i) the chaperoning capability of the sp^2-iminosugars 6S-NOI-NJ and 6S-NAdB-NJ is preserved upon complexation with $(ManS)_3$-βCD

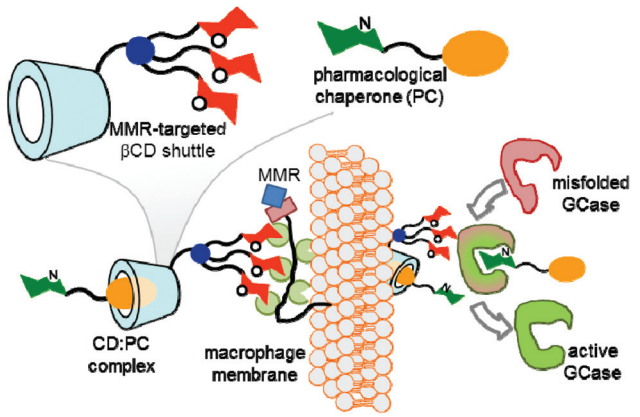


Fig. 2 Schematic representation of the strategy devised for macrophage-specific delivery of GD pharmacological chaperones by MMR-mediated mediated internalization of their inclusion complexes with the $(ManS)_3$-βCD carrier and further transfer to GCase.

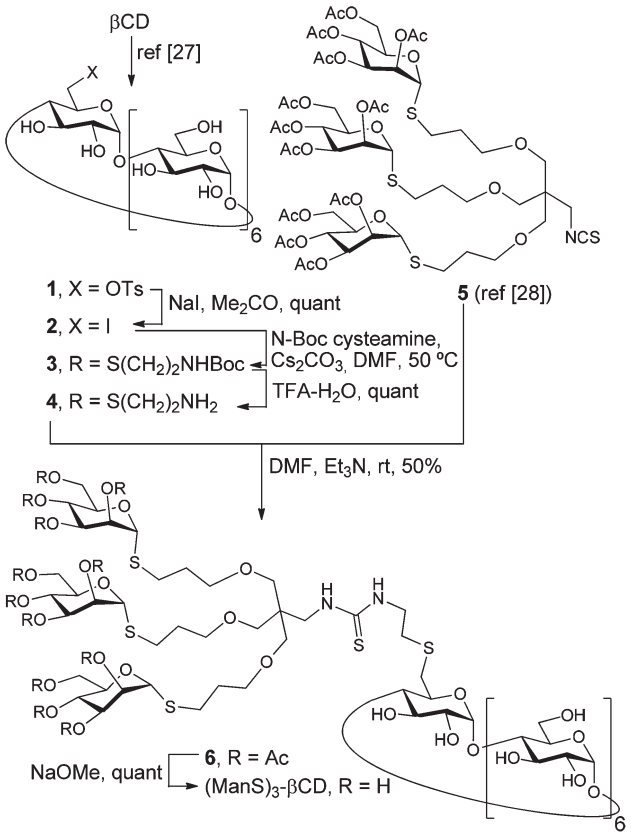
as determined in fibroblasts from GD patients and (ii) the PC-loaded βCD conjugates are internalized in macrophages, following the formation of PC:$(ManS)_3$-βCD:MMR ternary complexes, to finally deliver the PC inside the cells (Fig. 2).

Results and discussion

Selection criteria for the pharmacological chaperone and cyclodextrin carrier partners

Bicyclic 5*N*,6*S*-(*N*′-alkyliminomethylidene)-6-thionojirimycin derivatives have shown a strong and selective inhibitory activity against several β-glucosidases and excellent properties as pharmacological chaperones against several GD-associated GCase mutants.[15a] We have chosen the *N*′-octyl and the *N*′-[4-(adamant-1-ylcarboxamido)butyl] representatives 6S-NOI-NJ[22a] and 6S-NAdB-NJ[22b] in this study because the octyl and adamantyl moieties are known to exhibit a high avidity for the cavity of β-cyclodextrin in aqueous media, with association constant values (K_{as}) in the range 10^2–10^4 M^{-1}.[23] Inclusion complex formation with a βCD-based carrier is then expected to ensure efficient transfer of the PC from the CD cavity to the active site of GCase. In the design of $(ManS)_3$-βCD as the carrier partner for site-specific delivery of the selected PCs to macrophages, we kept in mind that multivalency is generally a prerequisite to elicit a biologically useful affinity in carbohydrate–protein recognition processes[24] and that monosubstitution of the βCD host by a mannosyl dendron is much less likely to alter the guest inclusion capabilities than polysubstitution strategies.[25]

The synthesis of $(ManS)_3$-βCD has been accomplished using a modular convergent strategy that takes advantage of the high efficiency of the thiourea-forming reaction for macromolecule conjugation[26] (Scheme 1). First, commercial βCD was regioselectively tosylated at a single primary O-6 position by reaction with *p*-toluenesulfonyl chloride in aqueous basic medium



Scheme 1 Building block-based synthesis of the CD carrier $(ManS)_3$-βCD.

in the presence of copper(II) (→**1**).[27] The incorporation of a cysteaminyl segment was next undertaken to avoid steric constrains during the final conjugation step and to warrant accessibility of the glycoligand to molecular recognition events in the final conjugate. Nucleophilic displacement of the tosylate group in **1** by 2-(*tert*-butoxycarbonylamino)ethanethiol (*N*-Boc-cysteamine) required harsh conditions and proved troublesome, however. Alternatively, tosylate was exchanged into iodine by treatment with NaI and the resulting iodo derivative **2** was next smoothly reacted with *N*-Boc-cysteamine to furnish adduct **3**. Acid-promoted carbamate removal quantitatively yielded the monocysteaminyl-functionalized βCD **4**. Coupling of **4** and the isothiocyanate-armed dendron **5**[28] was conducted in DMF under Et_3N catalysis at rt to give the corresponding thiourea adduct **6**. Final catalytic deacetylation quantitatively furnished the target $(ManS)_3$-βCD carrier, whose purity and structure were confirmed by mass spectrometry, NMR spectroscopy, and combustion analysis. The presence of the thiourea tether was ascertained by the ^{13}C NMR resonance at 183–180 ppm ($\delta_{C=S}$) and the typical line broadening associated with restricted rotation at the pseudoamide NH–(C=S) bond at room temperature.[29] The unsymmetrical nature of the cyclooligosaccharide resulted in extensive signal overlapping in the ^1H NMR spectrum that was partially overcome by using 1D TOCSY experiments.

$(ManS)_3$-βCD:pharmacological chaperone complex formation thermodynamics

To measure the affinity of the pharmacological chaperones 6S-NOI-NJ and 6S-NAdB-NJ for the $(ManS)_3$-βCD host, NMR titration experiments in D_2O were conducted. The resonances of the βCD H-3 and H-5 protons were the most intensely affected after complex formation, supporting inclusion of the hydrophobic moieties of the PCs in the βCD cavity. Using the continuous variations method and an iterative least squares fitting procedure[30] the corresponding association constant (K_{as}) values were determined to be 399 ± 4 and 1019 ± 196 M^{-1}, respectively (see the Experimental section and ESI† for details and binding isotherm figures). Titration isotherms were compatible with a 1 : 1 complex stoichiometry in both cases.

Glycosidase inhibition capabilities of $(ManS)_3$-βCD: pharmacological chaperone complexes

To pinpoint whether or not $(ManS)_3$-βCD complexation alters the availability of the pharmacological chaperones to interact with glycosidases, the inhibition capabilities of 6S-NOI-NJ and 6S-NAdB-NJ free or in complex with $(ManS)_3$-βCD were first profiled towards two commercial β-glucosidases (β-Glcases), namely β-Glcase from almonds and β-Glcase from bovine liver, belonging to the same clan as that of the human GCase (clan A).[31] Complexes were pre-formed by freeze-drying equimolecular mixtures of each sp^2-iminosugar and the mannosylated CD carrier $(ManS)_3$-βCD in water. The corresponding inhibition constants (K_i) at the optimal pH of each glycosidase are shown in Table 1. In control experiments, $(ManS)_3$-βCD alone did not affect the activities of the enzymes up to mM concentrations (data not shown). The inhibition mode, as determined by Lineweaver–Burk plots, was competitive in all cases.

The inhibitory activity of the pharmacological chaperones was not significantly altered after inclusion complex formation, with K_i values, in the μM range, that remained 2–3 orders of magnitude lower than the 6S-NOI-NJ : $(ManS)_3$-βCD or 6S-NAdB-NJ : $(ManS)_3$-βCD complex dissociation constants. Moreover, the percentage of enzyme inhibition for a constant concentration of 6S-NOI-NJ or 6S-NAdB-NJ was not affected by increasing concentrations of $(ManS)_3$-βCD up to 1 : 10 ratios. These results are in agreement with fast dynamics of the equilibrium at play that facilitates the efficient transfer of the 6S-NOI-NJ and 6S-NAdB-NJ chaperones from the CD cavity to the glycosidase active site. Further experiments with human GCase confirmed this hypothesis. Thus, the uncomplexed sp^2-iminosugars and the corresponding inclusion complexes with the $(ManS)_3$-βCD carrier were equally efficient at inhibiting glucocerebrosidase activity in cell lysates at concentrations over 0.1 μM (Fig. 3).

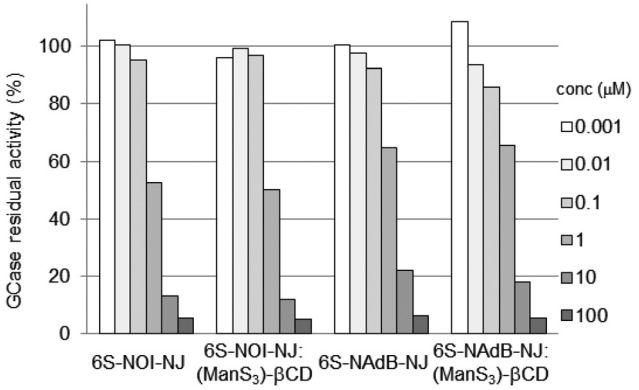


Fig. 3 Activity of wild-type human GCase at the indicated concentrations of the PCs 6S-NOI-NJ and 6S-NAdB-NJ or their corresponding 1 : 1 complexes with $(ManS)_3$-βCD. Results are expressed relative to activity of the enzyme in the absence of inhibitor (100%).

$(ManS)_3$-βCD:pharmacological chaperone complex formation thermodynamics

To probe the ability of the $(ManS)_3$-βCD carrier and the corresponding complexes with the 6S-NOI-NJ and 6S-NAdB-NJ to target mannose-specific lectins, a comparative study of their binding capabilities towards the model plant lectin Concanavalin A (Con A) by isothermal titration calorimetry (ITC) was first conducted. Con A specifically recognizes α-D-mannopyranosides.[32] For the measurements, the unloaded carrier and the preformed 1 : 1 inclusion complexes with each PC were titrated into a solution of Con A at pH 7.4. ITC data for the binding were fitted using a single site model based on monomeric Con A. The stoichiometry (n) determined for the complexes of any of these species with the lectin was 1 : 1 (n = 1); *i.e.*, the mannosyl dendron in $(ManS)_3$-βCD interacts exclusively with one unit of Con A, irrespective of being unloaded or loaded with the PC. Inclusion of the PC in the βCD cavity did not significantly affect the lectin affinity, with dissociation constants (K_D) for the Con A:[$(ManS)_3$-βCD : 6S-NOI-NJ] and Con A:[$(ManS)_3$-βCD : 6S-NAdB-NJ] complexes in the 1.6–3.6 μM range (Fig. 4 and Table 2) as compared with 2.1 ± 0.7 μM for the Con A:$(ManS)_3$-βCD complex. These values are indicative of 20-to-80-fold affinity enhancements as compared with reported data for methyl α-D-mannopyranoside

Table 1 Inhibition constants (K_i, μM) for 6S-NOI-NJ and 6S-NAdB-NJ and their corresponding 1 : 1 complexes with $(ManS)_3$-βCD towards commercial glucosidases

Glucosidase (source, pH)[a]	6S-NOI-NJ	6S-NOI-NJ : $(ManS)_3$-βCD	6S-NAdB-NJ	6S-NAdB-NJ : $(ManS)_3$-βCD
β-Glcase (almond, 7.3)	0.76 ± 0.05	0.29 ± 0.02	0.45 ± 0.03	5.8 ± 0.05
β-Glcase (bovine liver, 7.3)	3.7 ± 0.1	9.9 ± 0.1	68 ± 2.0	33 ± 0.02

[a] K_i values were determined from the corresponding Lineweaver–Burk plots (see ESI for experimental details).

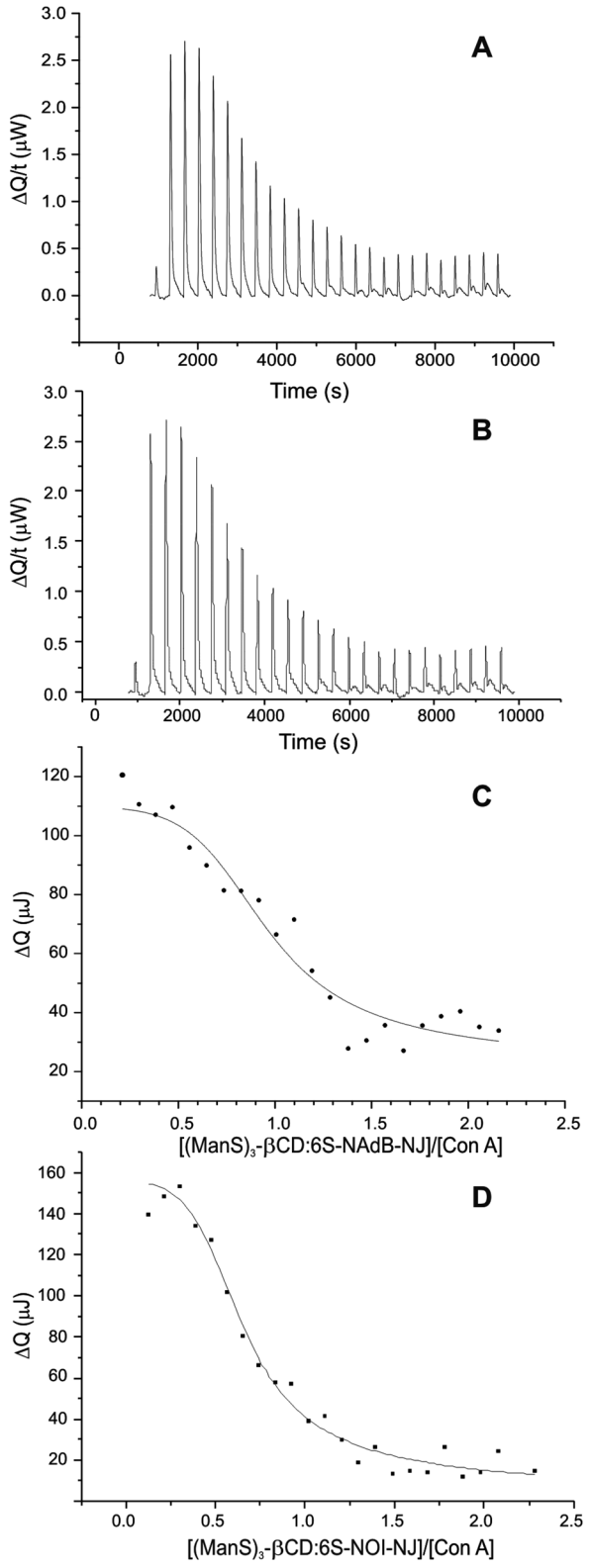


Fig. 4 Thermograms for the titration of Con A with the 1 : 1 $(ManS)_3$-βCD : 6S-NAdB-NJ and $(ManS)_3$-βCD : 6S-NOI-NJ complexes (A and B, respectively) and non-linear regression fittings to the 1 : 1 stoichiometry model (C and D, respectively). ΔQ represents the heat evolved after each injection of the complex.

determined by the same technique (K_D = 83 μM).[33] Altogether the data confirm that the trivalent mannosyl ligand in the designed PC carrier $(ManS)_3$-βCD is available to participate in lectin receptor recognition phenomena, benefitting from the multivalent effect, and that the carbohydrate–lectin interaction remains equally efficient after loading with the PC cargo.

Lectins recognizing an identical sugar epitope can quite differ structurally. Therefore, extrapolations of data on their responsiveness towards multivalent presentations of the putative ligand must be taken with care. We have made use of enzyme-linked lectin assay (ELLA) protocols, available for both Con A[34] and commercially available recombinant human macrophage mannose receptor (rhMMR),[35] to validate the results for macrophage targeting purposes. A 15-fold affinity enhancement against Con A for $(ManS)_3$-βCD as compared to the monovalent reference methyl α-D-mannopyranoside, expressed as the ratio between the concentrations required to achieve 50% inhibition (IC_{50}) of the association of horse radish peroxidase-labelled Con A (HRP-Con A) to an immobilized ligand (yeast mannan), was determined by ELLA, in agreement with literature data for other trivalent mannosides.[21a,28,36] In the case of the rhMMR, the ELLA experiment afforded a $(ManS)_3$-βCD *versus* methyl α-D-mannopyranoside relative affinity enhancement significantly higher (72-fold), suggesting that the disposition of the mannosyl epitopes in the dendron is particularly appropriate to benefit from the multivalent effect in the case of this lectin. Actually, control experiments indicated that the affinity of $(ManS)_3$-βCD towards rhMMR is analogous to that of high mannose oligosaccharides, known to be preferred ligands for this receptor in Nature,[36] in the same experimental setup (see ESI†).

Evaluation of the chaperoning capabilities of $(ManS)_3$-βCD : 6S-NOI-NJ and $(ManS)_3$-βCD : 6S-NAdB-NJ complexes in Gaucher fibroblasts

The GCase chaperoning capabilities of the sp^2-iminosugars 6S-NOI-NJ and 6S-NAdB-NJ before and after complexation with $(ManS)_3$-βCD were evaluated in three different mutant GD fibroblast lines, namely F213I/F213I (catalytic domain, neuronopathic), N370S/N370S (catalytic domain, non-neuronopathic) and L444P/L444P (non-catalytic domain, neuronopathic), as well as in wild-type fibroblasts. Cells were cultured in the absence and in the presence of different amounts of the PCs (3 and 30 μM) and their corresponding 1 : 1 complexes with $(ManS)_3$-βCD. After 4-day incubation, GCase activity was determined by *in situ* fluorimetric measurement of the production of 4-methylumbelliferone (see ESI† for details). As observed in Fig. 5A, neither 6S-NOI-NJ, 6S-NAdB-NJ nor their complexes disrupted the activity of the wild-type enzyme, revealing that lysosomal function is not inhibited at the highest concentration tested (30 μM). Both 6S-NOI-NJ and 6S-NAdB-NJ enhanced the activity of GCase in a dose dependent manner, up to 3-fold, in F213I/F213I mutant fibroblasts (Fig. 5B). The non-neuronopathic N370S/N370S mutant was much less sensitive to these PCs; only 6S-NAdB-NJ at 3 μM concentration was able to promote a significant enzyme activity

Table 2 Thermodynamic parameters and dissociation constant (K_D) calculated from ITC experiments for the binding of Con A to ($ManS_3$)-βCD and to the corresponding complexes with the pharmacological chaperones 6S-NOI-NJ and 6S-NAdB-NJ

	$\Delta G°$ (kJ mol^{-1})	$\Delta H°$ (kJ mol^{-1})	$T\Delta G°$ (kJ mol^{-1})	K_D (μM)
$(ManS)_3$-βCD	−32.4 ± 1.0	−137.7 ± 10.5	−105.2 ± 10.5	2.1 ± 0.7
6S-NOI-NJ : $(ManS)_3$-βCD	−33.0 ± 3.1	−125.3 ± 10.9	−92.3 ± 10.9	1.6 ± 0.1
6S-NAdB-NJ : $(ManS)_3$-βCD	−31.0 ± 1.3	−105.8 ± 2.4	−74.7 ± 2.4	3.6 ± 1.5

enhancement (about 2-fold; Fig. 5C). Negligible effects were measured in the L444P/L444P mutant with either 6S-NOI-NJ or 6S-NAdB-NJ, Fig. 5D. These results are consistent with the mutation-dependent responsiveness previously encountered for this family of PCs.[15a] In any case, the behavior of the two PCs was largely independent of inclusion complex formation with $(ManS)_3$-βCD, which is in agreement with a fast transfer of the PC from the βCD cavity in the carrier to the GCase active site.

Macrophage-targeting and delivery capabilities of $(ManS)_3$-βCD:pharmacological chaperone complexes

To confirm the potential of the $(ManS)_3$-βCD carrier to target the MMR in macrophages, the ability to adhere to resident peritoneal macrophages (mice) was first probed *in vitro*. Adhesion was quantified using a method adapted from that reported by Muller and Schuber[37] that takes advantage of the strong enhancement in the fluorescent intensity of 6-*p*-toluidino-2-naphthalenesulfonic acid (TNS) upon inclusion in the hydrophobic cavity of βCD and βCD derivatives to form 1 : 1 complexes.[21a] The cells were incubated with different concentrations of the 1 : 1 TNS : $(ManS)_3$-βCD complex at 4 °C to prevent phagocytosis and the amount of complex associated with the cell membrane was determined fluorimetrically. As denoted in Fig. 6A, fluorescence levels increased upon incubation of the macrophages with the TNS : $(ManS)_3$-βCD complex in a dose dependent manner, while the non-targeted TNS : βCD complex, used as a control, did only produce background fluorescence levels. Interestingly, TNS : $(ManS)_3$-βCD adhesion to macrophages was gradually inhibited in the presence of increasing concentrations of uncomplexed $(ManS)_3$-βCD or the corresponding 1 : 1 $(ManS)_3$-βCD : 6S-NOI-NJ and $(ManS)_3$-βCD : 6S-NAdB-NJ complexes with very similar efficiencies (Fig. 6B, IC_{50} 42, 39 and 36 μM, respectively). This result supports that the carrier and the corresponding PC or TNS complexes compete for the same receptor at the surface of macrophages and is in agreement with the involvement of the MMR in the adhesion process.

We have previously used a covalently attached dansyl tag to trace sp^2-iminosugar internalization in fibroblasts.[15b] However, the presence of the relatively big fluorescent moiety may significantly alter the membrane-crossing and diffusion properties of the molecule as compared with the unlabelled chaperone, limiting the scope of the conclusions. In order to ascertain whether or not the PC:$(ManS)_3$-βCD complexes are internalized in macrophages following MMR binding, we have instead devised competitive assays using the adamantane-equipped dansyl fluorescent probe **7** and the mannosyl derivative **8** (Fig. 7; see ESI† for synthetic details) as a model βCD guest and an MMR ligand, respectively. Experiments were conducted in macrophage-like cells differentiated from THP-1 human monocytes, monitoring by 3D fluorescence microscopy. These cells mimic many characteristic features of human primary macrophages, including MMR expression.

In control experiments, incubation of the cells with the reference compound **8** (400 μM) produced an intense green fluorescence in the cytoplasm that was virtually abolished by excess yeast mannan (data not shown), in agreement with MMR-mediated internalization of the conjugate. A virtually identical result was obtained when **8** was replaced by the 1 : 1 $(ManS)_3$-βCD : **7** complex at the same concentration (Fig. 8, row A), but not when uncomplexed **7** was incubated with the cells under identical conditions, strongly supporting that the $(ManS)_3$-βCD carrier promotes internalization of the included guest upon binding to the MMR. In parallel experiments, an excess of the sp^2-iminosugar 6S-NAdB-NJ did not affect internalization of **8**, discarding the direct interaction of the chaperone with the MMR. However, in the case of the 1 : 1 $(ManS)_3$-βCD : **7** complex the fluorescence intensity significantly decreased upon mixing with increasing amounts of 6S-NAdB-NJ prior to incubation of the cells (Fig. 8, rows B and C). This is consistent with the partial displacement of the fluorescent probe **7** from the carrier by the pharmacological chaperone and the subsequent internalization of the non-fluorescent $(ManS)_3$-βCD : 6S-NAdB-NJ complex through the MMR-mediated route. Accordingly, internalization of the $(ManS)_3$-βCD : **7** complex was also abolished in the presence of excess of yest mannan (Fig. 8, row D). A quantitative representation of the relative intracellular fluorescent intensities for the above experiments is shown in Fig. 8E. With the intrinsic limitations of any indirect method, the ensemble of results support the hypothesis that $(ManS)_3$-βCD specifically interacts with the MMR through the trivalent mannosyl antenna and that this interaction elicits internalization of the corresponding inclusion complexes with PCs into the macrophages.

Conclusions

The present study provides a proof of concept of the suitability of the βCD-mannosyl dendron conjugate $(ManS)_3$-βCD as a macrophage-targeted delivery system for sp^2-iminosugar-type amphiphilic pharmacological chaperones against Gaucher disease. The monosubstituted but multivalent conjugate structure of the carrier prototype has been purposely conceived to keep the inclusion capabilities towards the hydrophobic

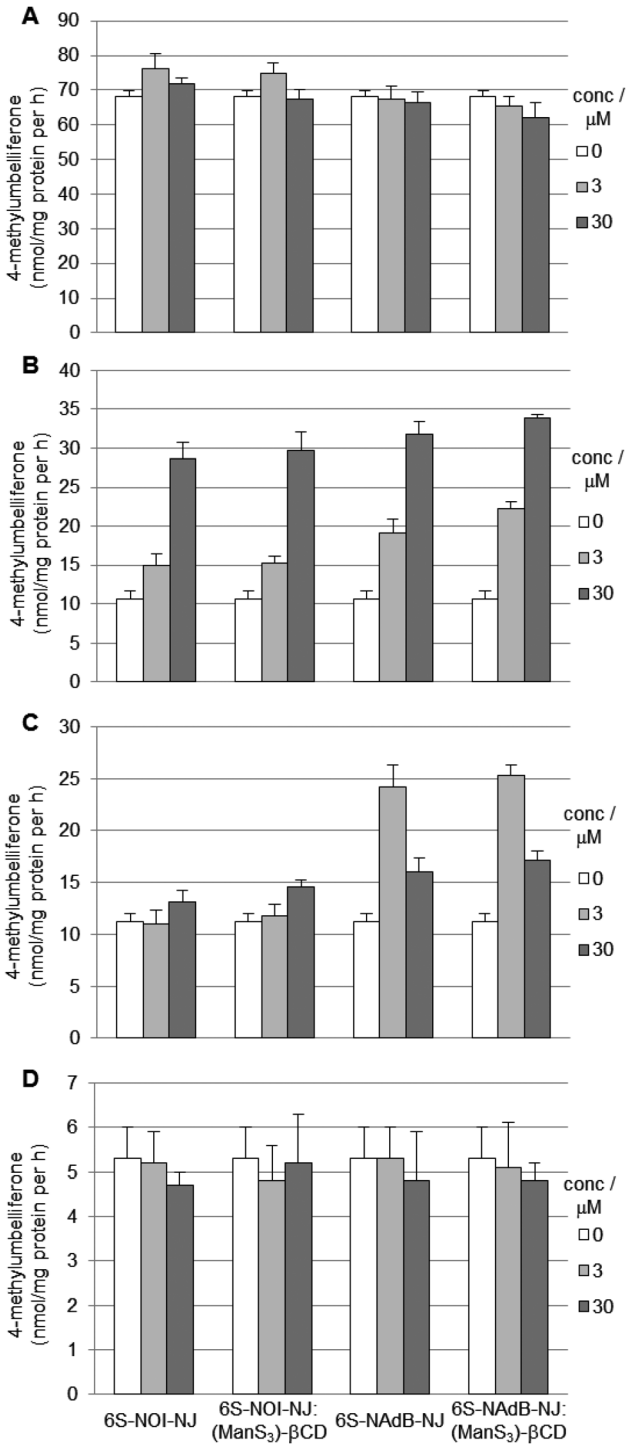


Fig. 5 Chaperone activity of 6S-NOI-NJ and 6S-NAdB-NJ and their corresponding 1 : 1 complexes with $(ManS)_3$-βCD on wild type (A) and mutant GCases (B, F213I/F213I; C, N370S/N370S; D, L444P/L444P) in fibroblasts (*in situ* cell enzyme assay). Cells were cultured for four days in the absence or presence of increasing concentrations of the compounds. Lysosomal GCase activity was estimated in intact cells by measuring the rate of production of 4-methylumbelliferone (see ESI† for details).

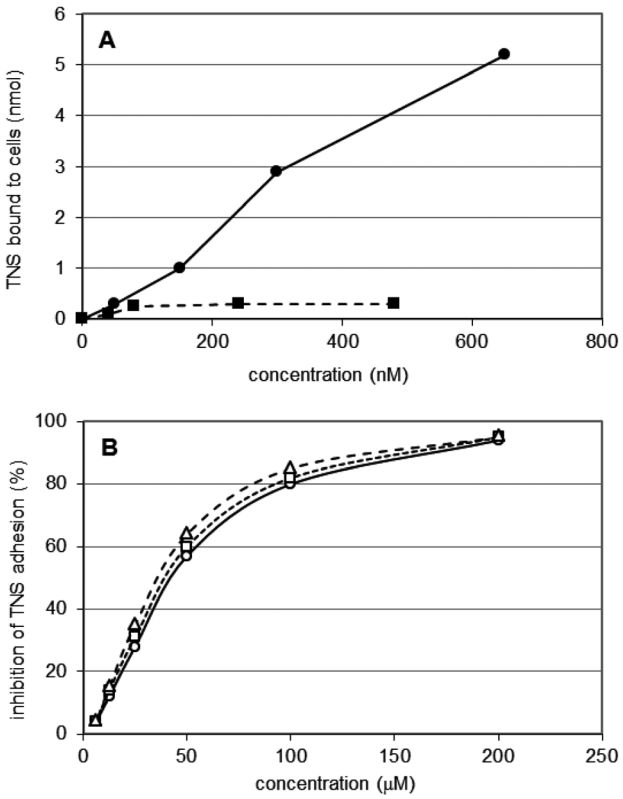


Fig. 6 (A) Fluorimetrically determined adhesion of 1 : 1 TNS : $(ManS)_3$-βCD (filled line, ●) and TNS : βCD (slashed line, <) complexes to the cell membrane of mice macrophages and (B) competitive membrane-bound TNS displacement assay using $(ManS)_3$-βCD (filled line, ○), and the 1 : 1 6S-NOI-NJ : $(ManS)_3$-βCD (dotted line, □) and 6S-NAdB-NJ : $(ManS)_3$-βCD (slashed line, Δ) complexes.

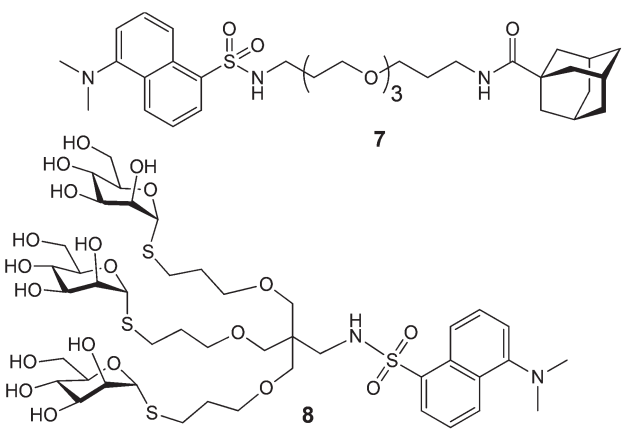


Fig. 7 Structure of dansylated probes **7** and **8** used for confocal microscopy monitoring of macrophage targeting and cellular uptake of PC:CD complexes.

moieties of the PC candidates, 6S-NOI-NJ and 6S-NAdB-NJ while warranting efficient mannose-specific lectin recognition abilities. Both hypotheses have been first demonstrated for commercial β-glucosidases and Con A lectin and further validated in human β-glucocerebrosidase and human MMR (recombinant). Complexation of the pharmacological

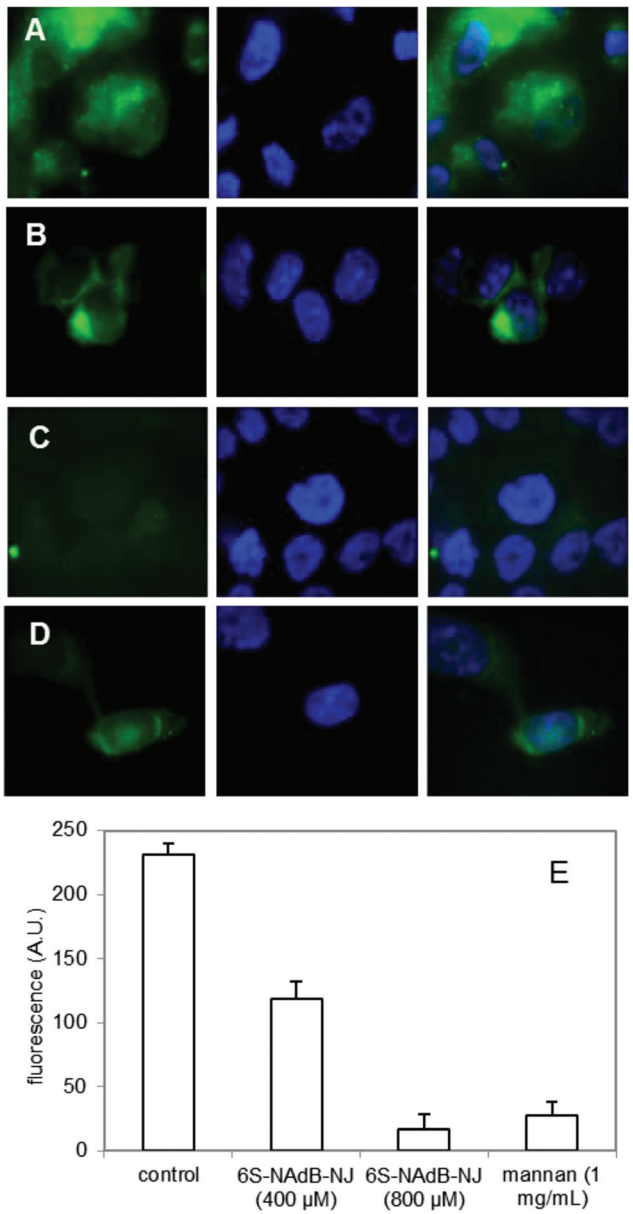


Fig. 8 Fluorescence microscopy pictures (3D projections) of THP-1 human monocytic cells differentiated into macrophage-like cells incubated with $(ManS)_3$-βCD:**7** complex (400 μM) alone (row A) and in the presence of 6S-NAdB-NJ (400 or 800 μM, rows B and C, respectively) or yeast mannan (1 mg mL^{-1}, row D). The fluorescence of probe **7**, the nuclear staining reagent Hoechst 33342, and the merged pictures are represented in the left, center, and right columns, respectively. Acquired *z* planes were separated by 0.3 μm, and an average of 50 planes was taken for each cell. Panel (E) quantitates the intracellular fluorescence after treatment with $(ManS)_3$-βCD : **7** complex (400 μM) alone (control) and in the presence of 6S-NAdB-NJ (400 or 800 μM) or yeast mannan (1 mg mL^{-1}). Identical circular regions of interest (ROIs) comprising 200 cells were taken for fluorescence quantification.

chaperones by $(ManS)_3$-βCD preserves their chaperoning potential. The corresponding PC complexes specifically recognize the MMR at the surface of macrophages, the cell type that is mostly affected in GD patients, and this recognition phenomenon elicits macrophage internalization. The combination of macrophage targeting abilities and efficient PC transfer to GCase may provide a means for improving the therapeutic outcome of pharmacological chaperone treatments for Gaucher disease and warrants further *in vivo* evaluation.

Experimental

General methods

6^I-*O*-*p*-Toluenesulfonylcyclomaltoheptaose (**1**)[27] and 2,2,2-tris-[5-(2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosylthio)-2-oxapentyl]-ethyl isothiocyanate (**5**)[28] were obtained according to literature procedures. Iminosugars 6S-NOI-NJ[22a] and 6S-NAdB-NJ[22b] were synthesized according to literature procedures. The synthesis of dansylated derivatives **7** and **8** is described in the ESI.† Optical rotations were measured at 20 ± 2 °C in 1 dm tubes on a Jasco P-2000 polarimeter. UV spectra were recorded in 1 cm tubes on a Jasco V-630 spectrophotometer. ^1H (and ^{13}C NMR) spectra were recorded at 500 (125.7) MHz with Bruker 500 DRX magnet. 1D TOCSY, 2D COSY, HMQC and HSQC experiments were used to assist on NMR assignments. Thin-layer chromatography (TLC) was carried out on aluminum sheets coated with silica gel 60 F254 Merck with visualization by UV light and by charring with ethanolic 10% H_2SO_4 and 0.1% ninhydrin. Column chromatography was carried out on Silica Gel 60. ESI mass spectra were recorded on a Bruker Daltonics Esquire6000™ ion-trap mass spectrometer. Elemental analyses were carried out at the Instituto de Investigaciones Químicas (Sevilla, Spain).

6^I-Deoxy-6^I-iodocyclomaltoheptaose (2).[38] To a solution of monotosylated βCD derivative 1 (1.0 g, 0.76 mmol) in DMF (18 mL) NaI (0.57 g, 3.8 mmol, 5 eq.) was added. The mixture was stirred at 80 °C overnight and then the solvent was removed under reduced pressure. The resulting residue was stirred in a mixture of ^{t}BuOH–EtOH–H_2O (5 : 4 : 3, 10 mL), filtrated and washed with cold EtOH (10 mL), DCM (10 mL) and acetone (10 mL), and dried to yield compound **2** in 90% yield (0.87 g). R_f = 0.18 (6 : 3 : 1 MeCN–H_2O–NH_4OH); $[\alpha]_D$ = +128.7 (*c* 0.75, MeOH); ^1H NMR (500 MHz, DMSO-d_6, 313 K): δ 5.72–5.60 (m, 13H, OH-$2^{I–VII}$, OH-$3^{II–VII}$), 5.56 (d, 1H, $J_{OH,3}$ = 2.3 Hz, OH-3^I), 4.89–4.83 (m, 6H, H-$1^{II–VII}$), 4.88 (d, 1H, $J_{1,2}$ = 3.7 Hz, H-1^I), 4.57–4.43 (m, 6H, OH-$6^{II–VII}$), 3.80–3.51 (m, 24H, H-$3^{II–VII}$, H-$5^{II–VII}$, H-$6^{II–VII}$), 3.70 (m, 1H, H-5^I), 3.63 (bd, 1H, $J_{6a,6b}$ = 11.3 Hz, H-$6a^I$), 3.54 (bd, 1H, $J_{5,6a}$ = 3.0 Hz, H-$6b^I$), 3.43 (m, 1H, H-3^I), 3.45–3.26 (m, 12H, H-$2^{II–VII}$, H-$4^{II–VII}$), 3.33 (m, 1H, H-2^I), 3.20 (t, 1H, $J_{3,4}$ = $J_{4,5}$ = 8.9 Hz, H-4^I); ^{13}C NMR (125.7 MHz, DMSO-d_6, 313 K): δ 102.7–102.1 (C-$1^{I–VII}$), 86.5 (C-4^I), 82.3–81.9 (C-$4^{II–VII}$), 73.6–72.4 (C-$2^{I–VII}$, C-$3^{II–VII}$ C-$5^{I–VII}$), 70.0 (C-3^I), 60.6–60.4 (C-$6^{II–VII}$), 10.0 (C-6^I); ESIMS: *m*/*z* 1279.7 $[M + Cl]^-$. Anal. Calcd for $C_{42}H_{69}IO_{34}$: C, 40.52; H, 5.59; found: C, 40.13; H, 5.62.

6^I-[2-(*tert*-Butoxycarbonylamino)ethylthio]cyclomaltoheptaose (3). To a solution of **2** (1.42 g, 1.14 mmol) and Cs_2CO_3 (0.48 g, 1.48 mmol, 1.3 eq.) in dry DMF (18 mL), under an Ar atmosphere, *tert*-butyl *N*-(2-mercaptoethyl)carbamate (250 μL, 1.48 mmol, 1.3 eq.) was added. The suspension was heated at

75 °C for 3 h and the solvent was reduced until 1/3 volume. The resulting residue was poured into acetone (150 mL), filtered and purified by column chromatography (6 : 3 : 1 MeCN–H_2O–NH_4OH) to give **3** in 67% yield (0.98 g); R_f = 0.47 (6 : 3 : 1 MeCN–H_2O–NH_4OH); $[\alpha]_D$ = +115.3 (*c* 1.0 in DMSO); ^{1}H NMR (500 MHz, 9 : 1 DMSO-d_6–D_2O, 333 K): δ 4.88–4.82 (m, 6H, H-1$^{II–VII}$), 4.84 (d, 1H, $J_{1,2}$ = 3.0 Hz, H-1^{I}), 3.86–3.59 (m, 24H, H-3$^{II–VII}$, H-5$^{II–VII}$, H-6$^{II–VII}$), 3.82 (bt, 1H, $J_{4,5}$ = $J_{5,6a}$ = 8.6 Hz, H-5^{I}), 3.65, (t, 1H, $J_{2,3}$ = 8.6 Hz, H-3^{I}), 3.42–3.26 (m, 12H, H-2$^{II–VII}$, H-4$^{II–VII}$), 3.34 (dd, 1H, H-2^{I}), 3.26 (t, 1H, H-4^{I}), 3.08 (m, 2H, CH_2N_{Cyst}), 3.07 (bd, 1H, H-6a^{I}), 2.71 (m, 1H, H-6b^{I}), 2.64 (m, 2H, CH_2S_{Cyst}), 1.43 (bs, 9H, CMe_3); ^{13}C NMR (100.6 MHz, 9 : 1 DMSO-d_6–D_2O): δ 156.0 (CO), 102.9–101.2 (C-1$^{I–VII}$), 86.7 (C-4^{I}), 82.3–82.0 (C-4$^{II–VII}$), 78.6 (C_q), 73.5–72.4 (C-2$^{I–VII}$, C-3$^{I–VII}$, C-5$^{I–VII}$), 60.6–60.2 (C-6$^{II–VII}$), 43.0 (CH_2N_{Cyst}), 39.9 (CH_2S_{Cyst}, C-6^{I}), 29.0 (CMe_3); ESIMS: *m*/*z* 1316.9 [M + Na]+. Anal. Calcd for $C_{49}H_{83}NO_{36}S$: C 45.47, H 6.46, N 1.08. Found: C 45.31, H 6.46, N 0.96.

6^{I}-(2-Aminoethylthio)cyclomaltoheptaose hydrochloride (4).[39] Compound **3** (94 mg, 72 μmol) was treated with a 1 : 1 TFA–water mixture (3 mL) at rt for 1 h. The solvents were then evaporated under reduced pressure and acid traces were removed by co-evaporating with water several times. The residue was finally dissolved in diluted aq. HCl and freeze-dried to quantitatively furnish compound **4** as hydrochloride salt (89 mg). $[\alpha]_D$ = +113.6 (*c* 0.9, H_2O); ^{1}H NMR (500 MHz, D_2O): δ 5.04 (d, 1H, $J_{1,2}$ = 3.7 Hz, H-1^{II}), 4.99 (d, 1H, $J_{1,2}$ = 3.7 Hz, H-1^{I}), 5.00–4.97 (m, 5H, H-1$^{III–VII}$), 3.95 (bt, 1H, $J_{4,5}$ = 9.4 Hz, H-5^{I}), 3.91–3.72 (m, 20H, H-3$^{III–VII}$, H-5$^{III–VII}$, H-6$^{III–VII}$), 3.86 (t, 1H, $J_{2,3}$ = $J^{3,4}$ = 9.4 Hz, H-3^{II}), 3.85 (t, 1H, $J_{2,3}$ = $J_{3,4}$ = 9.4 Hz, H-3^{I}), 3.78 (m, 2H, H-6^{II}), 3.77 (m, 1H, H-5^{II}), 3.61–3.44 (m, 12H, H-2$^{III–VII}$, H-4$^{III–VII}$), 3.58 (dd, 1H, H-2^{II}), 3.57 (m, 2H, H-2^{I}, H-4^{I}), 3.47 (t, 1H, $J_{4,5}$ = 9.4 Hz, H-4^{II}), 3.16 (t, 2H, $^3J_{H,H}$ = 6.4 Hz, CH_2N_{Cyst}), 3.07 (dd, 1H, $J_{5,6a}$ = 2.9 Hz, $J_{6a,6b}$ = 14.0 Hz, H-6a^{I}), 2.91 (dd, 1H, $J_{5,6a}$ = 6.5 Hz, H-6b^{I}), 2.86 (t, 2H, CH_2S_{Cyst}); ^{13}C NMR (125.7 MHz, D_2O): δ 101.9–101.7 (C-1$^{I–VII}$), 83.8 (C-4^{I}), 81.3–81.1 (C-4$^{II–VII}$), 73.0–71.1 (C-2$^{I–VII}$, C-3$^{I–VII}$, C-5$^{I–VII}$), 60.5–60.2 (C-6$^{II–VII}$), 38.4 (C-6^{I}), 32.5 (CH_2N_{Cyst}), 29.7 (CH_2S_{Cyst}); ESIMS: *m*/*z* 1194.8 $[M - Cl]^+$.

6^{I}-[2-[*N*′-[2,2,2-Tris[5-(2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosylthio)-2-oxapentyl]ethyl]thioureido]ethylthio]cyclomaltoheptaose (6). To a solution of **4** (94 mg, 72 μmol) and Et_3N (20 μL, 144 μmol, 2 eq.) in dry DMF (1 mL), a solution of **5** in dry DMF (1 mL) was added. The reaction mixture was stirred at room temperature for 72 h preserving the pH at 8–9 with Et_3N. The reaction mixture was then concentrated and the residue was purified by column chromatography (MeCN → 3 : 1 MeCN–H_2O) to give **6** in 50% yield (93 mg). R_f = 0.66 (6 : 3 : 1 MeCN–H_2O–NH_4OH); $[\alpha]_D$ = +57.8 (*c* 1.0, MeOH); ^{1}H NMR (500 MHz, CD_3OD, 313 K): δ 5.39 (bs, 3H, H-1$_{Man}$), 5.36 (d, 3H, $J_{1,2}$ = 1.6 Hz, $J_{2,3}$ = 2.8 Hz, H-2$_{Man}$), 5.30 (t, 3H, $J_{3,4}$ = $J_{4,5}$ = 9.8 Hz, H-4$_{Man}$), 5.23 (dd, 3H, H-3$_{Man}$), 5.04 (d, 1H, $J_{1,2}$ = 3.7 Hz, H-1^{II}), 5.01–4.98 (m, 5H, H-1$^{III–VII}$), 5.00 (d, 1H, $J_{1,2}$ = 3.3 Hz, H-1^{I}), 4.41 (ddd, 3H, $J_{5,6a}$ = 4.9 Hz, $J_{5,6b}$ = 2.1 Hz, H-5$_{Man}$), 4.30 (dd, 3H, $J_{6a,6b}$ = 12.1 Hz, $J_{5,6a}$ = 5.2 Hz, H-6a$_{Man}$), 4.16 (dd, 3H, $J_{5,6b}$ = 5.2 Hz, H-6b$_{Man}$), 3.97 (ddd, 1H, $J_{4,5}$ = 9.3 Hz, $J_{5,6a}$ = 2.3 Hz, $J_{5,6b}$ = 6.6 Hz, H-5^{I}), 3.92–3.84 (m, 17H, H-3$^{III–VII}$, H-6$^{II–VII}$), 3.88 (t, 1H, $J_{3,4}$ = 9.5 Hz, H-3^{II}), 3.87 (t, 1H, $J_{2,3}$ = $J_{3,4}$ = 9.3 Hz, H-3^{I}), 3.81–3.76 (m, 6H, H-5$^{II–VII}$), 3.73 (m, 4H, CH_2N, CH_2NH_{Cyst}), 3.58 (2d, 6H, $^3J_{H,H}$ = 6.0 Hz, H-3$_{Pent}$), 3.54 (dd, 1H, H-2^{I}), 3.54 (dd, 1H, $J_{1,2}$ = 9.5 Hz, H-2^{II}), 3.56–5.49 (m, 11H, H-2$^{III–VII}$, H-4$^{II–VII}$), 3.53 (m, 1H, H-4^{I}), 3.47 (m, 8H, CH_2NH, H-1$_{Pent}$), 3.18 (dd, 1H, $J_{6a,6b}$ = 12.8 Hz, H-6a^{I}), 2.94 (dd, 1H, H-6b^{I}), 2.88 (t, 2H, $^3J_{H,H}$ = 7.2 Hz, CH_2S_{Cyst}), 2.87–2.78 (m, 6H, H-5$_{Pent}$), 2.17–1.98 (4 s, 36H, MeCO), 1.96 (m, 6H, H-4$_{Pent}$); ^{13}C NMR (125.7 MHz, CD_3OD, 313 K): δ 182.0 (CS), 170.9–170.1 (CO), 102.5–102.2 (C-1$^{I–VII}$), 84.6 (C-4^{I}), 82.6 (C-1$_{Man}$), 81.8–81.7 (C-4$^{II–VII}$), 73.4–73.2 (C-3$^{I–VII}$), 72.9 (C-5$^{I–VII}$), 72.5–72.3 (C-2$^{I–VII}$), 71.1 (C-2$_{Man}$), 69.8 (C-3$_{Man}$), 69.5 (C-3$_{Pent}$), 69.1 (C-5$_{Man}$), 66.3 (C-4$_{Man}$), 62.4 (C-6$_{Man}$), 60.8–60.6 (C-6$^{II–VII}$, C-1$_{Pent}$), 44.3 (CH_2N, CH_2N_{Cyst}), 33.3 (C-6^{I}), 32.1 (CH_2S_{Cyst}), 29.3 (C-4$_{Pent}$), 28.0 (C-5$_{Pent}$), 19.4–19.1 (*Me*CO); ESIMS: *m*/*z* 1314.8 $[M + 2\ Na]^{2+}$. Anal. Calcd for $C_{101}H_{158}N_2O_{64}S_5$: C, 46.93; H, 6.16; N, 1.08; S, 6.20; found: C, 47.11; H, 6.34; N, 0.89; S, 5.87.

6^{I}-[2-[*N*′-[2,2,2-Tris[5-α-D-mannopyranosylthio)-2-oxapentyl]-ethyl]-thioureido]ethylthio]cyclomaltoheptaose ((ManS)$_3$-βCD). The trivalent mannosyl carrier $(ManS)_3$-βCD was obtained by treating a solution of compound **6** (59 mg, 23 μmol) in dry MeOH (3 mL) with methanolic NaOMe (1 m, 27 μL, 0.1 eq.) at rt for 1 h. The reaction mixture was neutralized with Amberlite IR-120 (H^+) ion exchange resin, the resin filtered-off and the solvent evaporated under reduced pressure to yield $(ManS)_3$-βCD in 97% yield (46 mg). $[\alpha]_D$ = +27.5 (*c* 1.0, H_2O); UV (H_2O) λ_{max} = 262 nm (ε_{mM} = 2308); ^{1}H NMR (500 MHz, D_2O, 323 K): δ 5.53 (bs, 3H, H-1$_{Man}$), 5.33 (m, 6H, H-1$^{II–VII}$), 5.29 (d, 1H, $J_{1,2}$ = 3.0 Hz, H-1^{I}), 4.29 (bs, 3H, H-2$_{Man}$), 4.19–3.99 (m, 31H, H-3$^{II–VII}$, H-5$^{II–VII}$, H-6$^{II–VII}$, H-1$_{Pent}$, H-3$_{Man}$, H-4$_{Man}$, H-5$_{Man}$, H-6$_{Man}$), 4.10 (t, 1H, $J_{2,3}$ = $J_{3,4}$ = 9.1 Hz, H-3^{I}), 4.09 (m, 1H, H-5^{I}), 3.97–3.85 (m, 12H, H-2$^{II–VII}$, H-4$^{II–VII}$), 3.93 (m, 2H, CH_2N_{Cyst}), 3.90 (dd, 1H, H-2^{I}), 3.76 (t, 6H, $^3J_{H,H}$ = 5.4 Hz, H-3$_{Pent}$), 3.75 (t, 1H, H-4^{I}), 3.65 (s, 2H, CH_2NH), 3.45 (bd, 1H, $J_{6a,6b}$ = 11.2 Hz, H-6a^{I}), 3.15 (t, 2H, $^3J_{H,H}$ = 6.5 Hz, CH_2S_{Cyst}), 3.07 (dd, 1H, $J_{5,6b}$ = 7.3 Hz, H-6b^{I}), 2.99–2.87 (m, 6H, H-5$_{Pent}$), 2.14 (m, 6H, H-4$_{Pent}$); ^{13}C NMR (125.7 MHz, D_2O, 323 K): δ 182.3 (CS), 102.6–102.1 (C-1$^{I–VII}$), 85.3 (C-1$_{Man}$), 85.0 (C-4^{I}), 81.3–80.9 (C-4$^{II–VII}$), 73.6–71.4 (C-2$_{Man}$, C-3$_{Man}$, C-5$_{Man}$, C-2$^{I–VII}$, C-3$^{I–VII}$, C-5$^{I–VII}$), 70.4 (C-3$_{Pent}$), 67.2 (C-4$_{Man}$), 61.5 (C-1$_{Pent}$), 60.5–60.2 (C-6$^{II–VII}$), 44.7 (CH_2N, CH_2N_{Cyst}), 34.2 (C-6^{I}), 33.8 (CH_2S_{Cyst}), 29.3 (C-4$_{Pent}$), 28.0 (C-5$_{Pent}$); ESIMS: *m*/*z* 1062.3 $[M + 2\ Na]^{2+}$. Anal. Calcd for $C_{77}H_{134}N_2O_{52}S_5$ C, 44.46; H, 6.49; N, 1.35; S, 7.71; found: C, 44.25, H, 6.22; N, 1.03; S, 7.42.

NMR titration experiments

Association constants (K_{as}) were determined in D_2O at 313 K by measuring the proton chemical shift variations in the ^{1}H NMR spectra of a solution of the βCD derivative $(ManS)_3$-βCD in the presence of increasing amounts of the corresponding iminosugar (6S-NOI-NJ or 6S-NAdB-NJ). The chemical shifts of the diagnostic signals obtained at 10–11 different CD:iminosugar concentration ratios were used in an iterative least-squares fitting procedure. For a detailed description, see ESI.†

Isothermal titration calorimetry (ITC)

ITC experiments were performed in a multichannel thermal activity monitor (TAM) isothermal heat conduction microcalorimeter (Thermometric AB 2277/201, Järfälla, Sweden) equipped with a 1.1 mL titration vessel. The calorimeter was thermostated at 25 ± 0.5 °C. The vessel was loaded with 0.8 mL of protein solution using a Hamilton syringe, thermostated at 25 °C and continuously stirred at 60 rpm. The CD derivative 3 was injected by a computer controlled syringe pump (Hamilton Microlab M). Injections were made over a period of 10 s with intervals of 6 min. The experiment was monitored and analyzed using Digitam 4.1 software (Thermometric). To minimize dilution artifacts, the ligand was dissolved in the same dialysis buffer as the protein. The errors are provided by the software from the best fit of the experimental data to the model of equal and independent sites, and correspond to the standard deviation in the fitting of the curves. A separate experiment was performed to determine the heat of dilution of the ligand in the dialysis buffer. Reported data are the mean of two measurements made with different protein preparations; experiments were repeated more than twice if the obtained parameters were not congruent.

Inhibition assays against commercial glycosidases

Inhibition constant (K_i) values were determined by spectrophotometrically measuring the residual hydrolytic activities of the β-glucosidases (from bovine liver or almonds; Sigma) against *p*-nitrophenyl β-D-glucopyranoside. Each assay was performed in phosphate buffer at the optimal pH for the enzymes. The reactions were initiated by addition of enzyme to a solution of the substrate in the absence or presence of various concentrations of iminosugar or iminosugar:CD complex (prepared by freeze-drying equimolecular mixtures of each partner). The mixture was incubated for 10–30 min at 37 °C and the reaction was quenched by addition of 1 M Na_2CO_3. Reaction times were appropriate to obtain 10–20% conversion of the substrate in order to achieve linear rates. The absorbance of the resulting mixture was determined at 405 nm. Approximate values of K_i were determined using a fixed concentration of a substrate (around the K_M value for the different β-glucosidases) and various concentrations of an inhibitor. Full K_i determinations and enzyme inhibition mode were determined from the slope of Lineweaver–Burk plots and double reciprocal analysis. Representative examples of the Lineweaver–Burk plots, with typical profile for competitive inhibition mode, are shown in the ESI (Fig. S11–S13†).

Lysosomal enzyme activity assay

Lysosomal enzyme activities in cell lysates were determined as described previously.[40] Briefly, cells were scraped in ice-cold 0.1% Triton X-100 in water. After centrifugation (6000 rpm for 15 min at 4 °C) to remove insoluble materials, protein concentrations were determined using a protein assay rapid kit (Wako, Tokyo, Japan). The lysates were incubated at 37 °C with the corresponding 4-methylumbelliferyl β-D-glycopyranoside solution in 0.1 M citrate buffer (pH 4). The liberated 4-methylumbelliferone was measured with a fluorescence plate reader (excitation 340 nm; emission 460 nm; Infinite F500, TECAN Japan, Kawasaki, Japan). For enzyme inhibition assay, cell lysates from normal skin fibroblasts were mixed with the 4-methylumbelliferyl β-D-glycopyranoside substrates in the absence or presence of increasing concentrations of the pharmacological chaperones 6S-NOI-NJ and 6S-NAdB-NJ or their corresponding 1 : 1 complexes with the mannosylated carrier $(ManS)_3$-βCD.

Cell culture and GCase activity enhancement assay

Human skin fibroblasts were cultured in Dulbecco's modified Eagle's medium–10% foetal bovine serum at 37 °C under a humidified atmosphere containing 5% CO_2. One control cell line (H37) and three lines of GD cells that carried the GlcCerase mutations F213I/F213I, L444P/L444P, and N370S/N370S, respectively, were used.[40] The culture medium was replaced every 2 d with fresh medium. For enzyme activity enhancement assay, cells were cultured in the presence of different concentrations of the pharmacological chaperones 6S-NOI-NJ and 6S-NAdB-NJ or their corresponding 1 : 1 complexes with the mannosylated carrier $(ManS)_3$-βCD for 5 days and harvested by scraping. Cytotoxicity of the compounds was monitored by measuring the lactate dehydrogenase activities in the cultured supernatants (LDH assay kit, Wako, Tokyo, Japan).

Enzyme-linked lectin assays (ELLA)

Nunc-Inmuno plates (MaxiSorp™) were coated overnight with yeast mannan at 100 μL per well diluted from a stock solution of 10 μg mL^{-1} in 10 mM phosphate buffer saline (PBS, pH 7.3 containing 0.1 mM Ca^{2+} and 0.1 mM Mn^{2+}) at rt. The wells were then washed three times with 300 μL of washing buffer (containing 0.05% (v/v) Tween 20) (PBST). The washing procedure was repeated after each of the incubations throughout the assay. The wells were then blocked with 150 μL per well of 1% BSA/PBS for 1 h at 37 °C.

For determination of Con A binding affinity, the wells were filled with 100 μL of serial dilutions of peroxidase labeled Con A from 10^{-1} to 10^{-5} mg mL^{-1} in PBS, and incubated at 37 °C for 1 h. The plates were washed and 50 μL per well of 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) (0.25 mg mL^{-1}) in citrate buffer (0.2 M, pH 4.0 with 0.015% H_2O_2) was added. The reaction was stopped after 20 min by adding 50 μL per well of 1 M H_2SO_4 and the absorbances were measured at 415 nm. Blank wells contained citrate-phosphate buffer. The concentration of lectin that displayed an absorbance between 0.8 and 1.0 was used for inhibition experiments. In order to carry out the inhibition experiments, each inhibitor was added in a serial of 2-fold dilutions (60 μL per well) in PBS with 60 μL of the desired lectin–peroxidase conjugate concentration on Nunclon™ (Delta) microtiter plates and incubated for 1 h at 37 °C. The above solutions (100 μL) were then transferred to the mannan-coated microplates, which were incubated for 1 h at 37 °C. The

plates were washed and the ABTS substrate was added (50 μL per well). Color development was stopped after 20 min and the absorbances were measured.

For determination of recombinant human MMR (rhMMR) binding affinity, the wells were filled with 100 μL of serial dilutions of rhMMR from a 10 mg mL^{-1} stock solution in PBS (pH 7.3 containing 0.1 mM Ca^{2+} and 0.1 mM Mn^{2+}), and incubated at 37 °C for 1 h. The plates were washed three times with PBST as described above and 100 μL of a solution of biotinylated anti-human MMR antibody (0.2 mg mL^{-1}; R&D Systems) in PBS was added in each well, and the plates were further incubated for 1 h at 37 °C. The complex NeutrAvidin®-biotinylated HRP was preformed separately by successively adding to Tris buffer (9.6 mL, 50 mM, pH 7.6) a solution of NeutrAvidin® (100 μg mL^{-1} in Tris buffer, 1.2 mL; Thermo Scientific) and a solution of biotin-conjugated HRP (25 μg mL^{-1} in Tris buffer, 1.2 mL; Thermo Scientific). The mixture was shaken for 30 min at rt and the solution was immediately transferred into the plates (60 μL per well). After 1 h at 37 °C, these plates were washed twice with Tris (250 μL per well) and ABTS (0.25 mg mL^{-1}, 50 μL per well) in citrate buffer (0.2 M, pH 4.0 with 0.015% H_2O_2) was added. After 5 min at rt, the optical density was measured at 415 nm. Blank wells were processed with anti-human MMR antibody as well as NeutrAvidin®-biotinylated HRP. The concentration of rhMMR that displayed an absorbance between 0.8 and 1.0 was used for inhibition experiments. For the competitive lectin binding inhibition experiment, $(ManS)_3$-βCD was mixed in a serial of 2-fold dilutions (60 mL per well) in HEPES buffer (20 mM, pH 7.4) with 60 mL of the appropriate rhMMR concentration in PBS buffer on Nunclon® (Delta) microtitre plates and incubated for 1 h at 37 °C. The above solutions (100 μL) were then transferred to the mannan-coated titer plates, which were incubated for 1 h at 37 °C. The plates were washed and the solution of biotinylated anti-human MMR antibody in PBS (100 μL) was added in each well, and the plates were further incubated for 1 h at 37 °C. Then the NeutrAvidin® solution was transferred into the plates (60 μL per well). After 1 h at 37 °C, these plates were washed twice with Tris (250 μL per well) and ABTS was added (50 μL per well). Optical density at 415 nm was determined after 5 min.

The percentage of inhibition was calculated as follows:

$$\%\ \text{Inhibition} = [A_{(\text{no inhibitor})} - A_{(\text{with inhibitor})}]/A_{(\text{no inhibitor})} \times 100$$

Results in triplicate were used for plotting the inhibition curves for each individual ELLA experiment. Typically, the IC_{50} values (concentration required to achieve 50% inhibition of the lectin association to the coating polysaccharide) obtained from several independently performed tests were in the range of ±15%. Nevertheless, the relative inhibition values calculated from independent series of data were highly reproducible. The inhibition for methyl α-D-gluco- and mannopyranoside were included as positive and negative controls, respectively.

Determination of mice macrophage adhesion

For evaluation of the interaction with macrophages, the procedure reported by Muller and Schuber[37] for mannosylated liposomes was adapted. Briefly, resident peritoneal macrophages were obtained from female Balb/c mice (6 to 8 weeks old) and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% decomplemented fetal calf serum (FCS) containing heparin (5 U mL^{-1}). The cell number was adjusted to 10^6 cells mL^{-1} and the suspension was plated (final volume 1 mL) in multiwell plates. After 2 h under a humidified atmosphere of 5% CO_2 in air (final pH 7.4), non-adherent cells were eliminated by rinsing the dishes three times with PBS. The adherent cells, 24 h after their isolation, were fed with fresh serum-less DMEM and incubated with different amounts of TNS : $(ManS)_3$-βCD complex. TNS : βCD complex was used as control. After the incubation time, the medium was pipetted-off and the cells washed four times with cold PBS (4 °C). TNS associated with the cells was determined fluorimetrically (JASCO fluorimeter, model FP-715, excitation at 308 nm and monitoring emission at 443 nm) after cell digestion in 1 mL PBS containing 0.1% of emulphogene BC-720, and scraped with a rubber policeman. Standard fluorescence curves were established under the same conditions with aliquots of the initial TNS : $(ManS)_3$-βCD and TNS : βCD preparations in order to correlate the measured fluorescence intensity with the amount of CD derivative. Experiments were performed in duplicate, and results did not differ more than 5%.

Human macrophage internalization monitoring by fluorescence microscopy

THP-1 human monocytic cells were cultured in RPMI medium supplemented with penicillin, streptomycin, and 10% fetal bovine serum at 37 °C in a humidified 5% CO_2 atmosphere. Cells were seeded onto 6-well plates at 1.5 × 3 10^6 cells per well. THP-1 monocytic cells were differentiated into macrophage-like cells by phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich) incubation at a final concentration of 100 ng mL^{-1} for 3 d and it was followed by 1 d in PMA-free medium before treatments.

THP-1 macrophages were grown on 1 mm (Goldseal No. 1) glass coverslips for 24 h in RPMI containing 10% fetal bovine serum. After treatment, cells were rinsed once with PBS, fixed in 3.8% paraformaldehyde for 5 min, and permeabilized in 0.1% saponin for 5 min. For nuclei staining, glass coverslips were then rinsed with PBS for 3 min, incubated for 1 min with PBS containing Hoechst 33 342 (1 mg mL^{-1}) and washed with PBS (three 5 min washes). Finally, the coverslips were mounted onto microscope slides using Vectashield Mounting Medium (Vector Laboratories, Burlingame, CA, USA). 3D projections were performed using a Delta-Vision system (Applied Precision; Issaquah, WA) with an Olympus IX-71 microscope (Shinjuku, Japan) with 100× objective/1.35 NA and filters set for DAPI, fluorescein isothiocyanate provided by Applied Precision. Acquired z planes were separated by 0.3 μm, and an

average of 50 planes was taken for each cell. The 3D stacks were subjected to Quick Projection using the Softworx software. This allows confirming that the fluorescence is located inside the cell and not just in the cell membrane. Quantification of fluorescence signal was performed in 200 cells using ImageJ software (National Institutes of Health, Bethesda, Maryland, USA).

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Trehalose- and Glucose-Derived Glycoamphiphiles: Small-Molecule and Nanoparticle Toll-Like Receptor 4 (TLR4) Modulators

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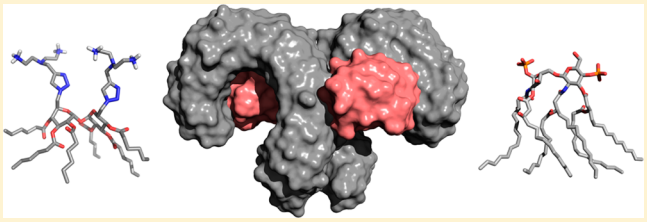
Supporting Information

ABSTRACT: An increasing number of pathologies have been linked to Toll-like receptor 4 (TLR4) activation and signaling, therefore new hit and lead compounds targeting this receptor activation process are urgently needed. We report on the synthesis and biological properties of glycolipids based on glucose and trehalose scaffolds which potently inhibit TLR4 activation and signaling in vitro and in vivo. Structure−activity relationship studies on these compounds indicate that the presence of fatty ester chains in the molecule is a primary prerequisite for biological activity and point to facial amphiphilicity as a preferred architecture for TLR4 antagonism. The cationic glycolipids here presented can be considered as new lead compounds for the development of drugs targeting TLR4 activation and signaling in infectious, inflammatory, and autoimmune diseases. Interestingly, the biological activity of the best drug candidate was retained after adsorption at the surface of colloidal gold nanoparticles, broadening the options for clinical development.



INTRODUCTION

Toll-like receptors (TLRs) play a critical role in the recognition of conserved pathogen-associated molecular patterns (PAMPs) derived from various microbial pathogens, including viruses, bacteria, protozoa, and fungi, and in the subsequent initiation of innate immune response.[1] Among TLRs, TLR4 selectively responds to bacterial endotoxin (E), composed of bacterial lipopolysaccharides (LPS) or part of it (lipooligosaccharides, LOS, lipid A).[2,3] LPS is the main chemical component of the Gram negative bacteria outer membrane, and the lipid A, a negatively charged phosphorylated lipodisaccharide represents the LPS moiety that is responsible for TLR4 activation through specific molecular recognition processes (Figure 1).

TLR4 is also activated by endogenous molecules, generally known as danger-associated molecular patterns (DAMPs).[4] Typical DAMPs acting as TLR4 agonists are released as a consequence of injury and inflammation. Most of the reported DAMPs are proteins, which are very different from lipid A, and the molecular details of DAMP interaction with the TLR4 receptorial system are still unknown, although in some cases endotoxin contamination seems to be responsible for TLR4 activity of DAMPs. Chemical entities that block TLR4 activation by bacterial endotoxin (LPS), thus acting as antagonists, are hit compounds for developing drugs active against acute sepsis and septic shock derived from excessive and deregulated TLR4 activation and signaling.[5] On the other hand, the inhibition of TLR4 stimulation by DAMPs could be used to contrast a wide range of inflammatory and autoimmune disorders associated with the release of inflammatory cytokines. In this context, TLR4 is an emerging molecular target related to an impressively broad spectrum of modern day disorders including autoimmune disorders, chronic inflammations, allergies, asthma, atherosclerosis, aortic aneurysm, CNS diseases such as neuropathic pain, amyotrophic lateral sclerosis (ALS), Alzheimer's disease (AD), and some types of cancer.[6] As the majority of these pathologies still lack specific pharmacological treatment, small molecules active in inhibiting TLR4 activation have attracted increasing interest in a wide range of possible clinical settings.[7]

The molecular mechanism by which endotoxin activate TLR4 is a complex process[8] and depends on LPS binding protein (LBP)[9]-catalyzed extraction and transfer of individual LPS molecules from aggregated LPS to the CD14 (cluster of differentiation 14) receptor[10] and then from CD14 to myeloid differentiation protein 2 (MD-2).[11,12] This process is followed

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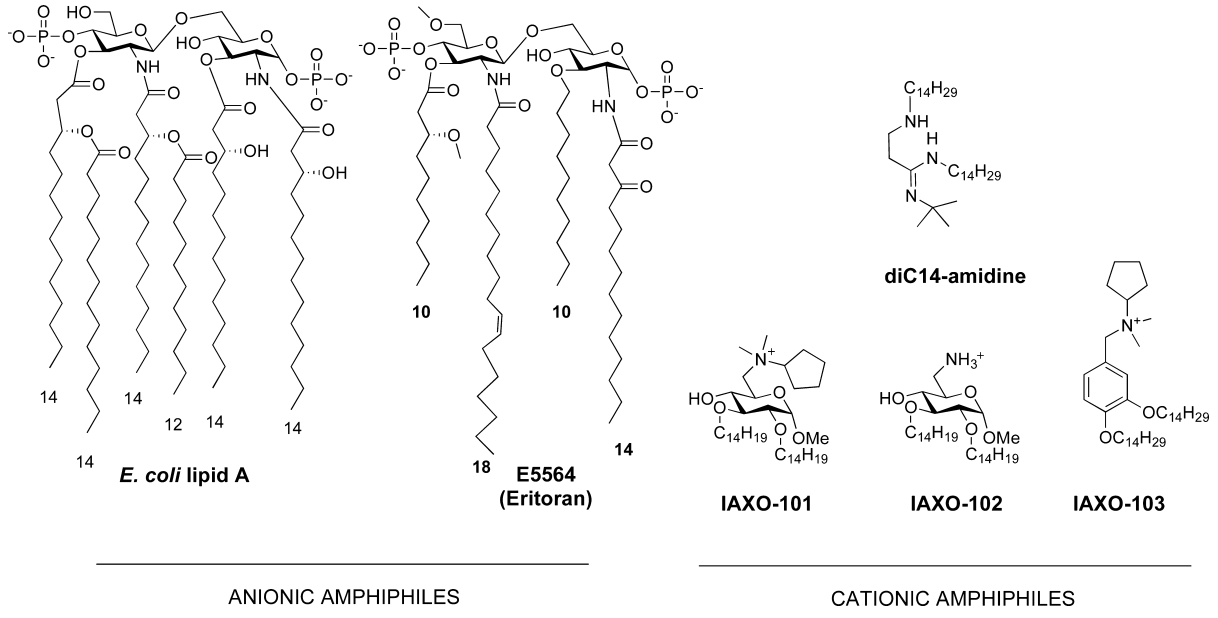

Figure 1. Anionic and cationic TLR4 modulators. From the left: lipid A from *Escherichia coli*, the natural TLR4 agonist, synthetic anionic (the antagonist Eritoran), and cationic amphiphiles (diC14-amidine, IAXO compounds).

by engagement and dimerization of TLR4, thus forming the cell surface complex $(LPS{\cdot}MD\text{-}2{\cdot}TLR4)_2$,[13] which initiates the intracellular signaling by recruiting specific adaptor proteins and activating downstream signaling pathways.

Several natural and synthetic small molecules are known to modulate TLR4 activation and subsequent intracellular signaling acting as agonists (activators) or antagonists (inhibitors).[6] The majority of these molecules are lipid A variants and synthetic lipid A mimetics that reproduce the structural motif of the anionic disaccharide. Monophosphoryl lipid A (MPL) and some aminoalkyl glucosaminide phosphates (AGPs) are agonists in use as vaccine adjuvants,[14] while underacylated variants such as natural lipid IVa[15] and synthetic Eritoran[16] are antagonists (Figure 1). In general, lipid A variants are anionic lipids, bearing one or two negatively charged phosphate groups and a hydrophobic domain (lipid chains). Although counterintuitive, several cationic lipids made of positively charged headgroups (usually tertiary or quaternary ammonium salts or polyamines) and a hydrophobic domain (alkyl chains or steroids) have been found to be active in modulating TLR4 activity,[17] acting either as agonists or antagonists of TLR4. Thus, some positively charged liposomes formed by cationic amphiphiles induce the expression of pro-inflammatory mediators. For instance, diC14-amidine (Figure 1) liposomes trigger the secretion of a cytokine pattern reminiscent of the TLR4-dependent LPS secretion pattern by activating both MyD88/NF-κB/JNK and TRAM/TRIF pathways.[18] Other cationic lipids activate cytokine production through NF-κB-independent, TRIF-dependent pathways, which requires the presence of CD14.[19,20] Structural changes make cationic lipids switch from agonism to antagonism, as in the case of dioleoyl trimethylammonium propane (DOTAP), that inhibits TLR4 signal by competing with LPS for interaction with LBP and/or CD14.[20] Complexes of the commercial cationic lipid formulation lipofectamine with LPS reduce its TLR4 activity. Interestingly LPS complexed with lipofectamine colocalizes with CD14 at the cell surface and inside cells but does not colocalizes with TLR4·MD-2 complex, suggesting that the mechanism of inhibition may result from the uncoupling of CD14 from TLR4·MD-2.[21]

Specific binding of amino glycolipids and aromatic ammonium salts to CD14 (compounds IAXO-101, -102, -103, Figure 1), was recently shown by our group.[22,23] These compounds are active in inhibiting LPS-stimulated TLR4-dependent cytokine production in cells and in animals.[24]

Evaluation of transfer of LOS from the monomeric soluble form of CD14 (sCD14) to His_6-tagged CD14 or MD-2 by cocapture to a metal chelating resin clearly showed that the cationic lipids derived from D-glucose or benzylamine inhibit the transfer of LOS from sCD14 to CD14-His_6, but not the transfer of LOS from sCD14 to MD-2.[22] Finally, saturation transfer difference (STD) NMR data demonstrated direct binding of the cationic lipids to CD14, mainly through alkyl chains.[22] Altogether, these data suggest that the lipid tails of cationic amphiphiles interact with the hydrophobic binding site of CD14[25] and compete with LPS or LOS chains. The carbohydrate scaffold in amino glycolipids probably acts by preventing random conformations and providing a favorable orientation of the lipid chains that is reminiscent of that found in lipid A. Most interestingly, through the interplay of regioselective functionalization methodologies and conformational bias, the installation of differentiated cationic and hydrophobic domains in carbohydrate platforms can be made compatible with molecular diversity-oriented strategies and structure−activity relationship (SAR) studies. As a proof of concept, we have now prepared a series of new cationic glycoamphiphiles using the monosaccharide methyl α-D-glucopyranoside and the disaccharide α,α′-trehalose as the sugar cores. Systematic modification of the cationic heads and the lipophilic tails and evaluation of their capacity to interfere with TLR4 activation and signaling in vitro and in vivo allowed the identification of a drug lead that has been further incorporated in gold nanoparticles to test the effect of multivalent presentation on its biological activity.

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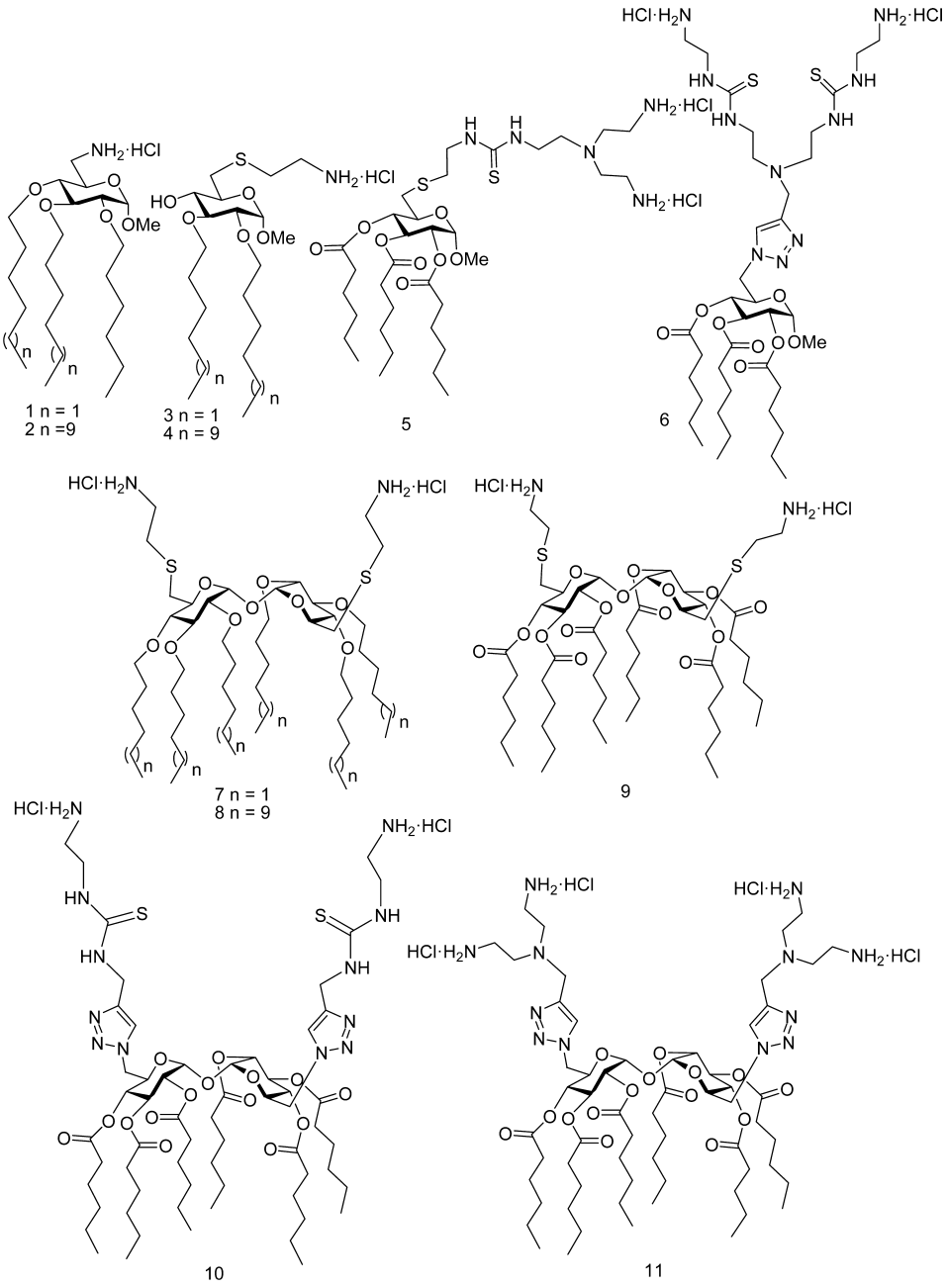

Figure 2. Synthetic monosaccharide and disaccharide protonatable amphiphiles derived, respectively, from D-glucose (**1**−**6**) and α,α'-trehalose (**7**−**11**).

RESULTS AND DISCUSSION

Ligand-Based Rational Design. It is known that the self-assembling capabilities and the ability of cationic amphiphiles to interact with CD14 as liposomes or micellar aggregates have a strong impact on their TLR4 modulatory activity.[17] Yet, very little is still known on the molecular aspects underlying the mechanisms at play, mainly because structural data of cationic compounds bound to MD-2 or CD14 receptors are still lacking. Conducting SAR studies on series of homologous cationic amphiphiles and relating the biological activity to the aggregation properties is expected to provide new insights in this matter. Glucose-derived cationic glycolipids are particularly appealing for this purpose. First, the secondary hydroxyls of the glucopyranose ring are well suited anchoring points to link lipophilic chains in a similar orientation as the fatty acid acyl chains in lipid A. Second, the incorporation of protonatable headgroups at the primary position imparts facial amphiphilicity to the molecule, a biomimetic feature that is associated with improved cell membrane crossing abilities and proneness to form supramolecular complexes with complementary biomolecules by either electrostatic or hydrophobic interactions.[26] On these grounds, we have now synthesized amino glycolipids derived from methyl α-D-glucopyranoside (**1**−**6**) and from α,α'-trehalose (**7**−**11**, Figure 2). The latter can be formally considered as dimeric homologues of the glucose amphiphiles. We keep in mind that the confluence of two exoanomeric effects at the 1−1 interglycosidic linkage in α,α'-trehalose strongly limits rotation about the glycosidic bonds,[27] preserving a rigid conformation that warrants facial anisotropy after differential functionalization at the primary and secondary

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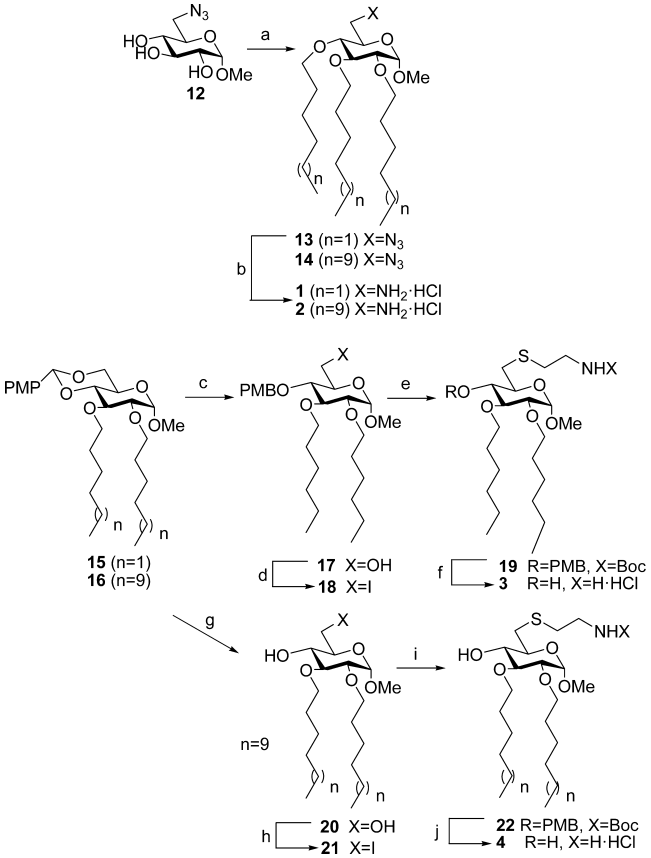
positions, even in highly constrained constructs.[28] Both the methyl α-D-glucopyranoside and the α,α′-trehalose scaffolds have previously demonstrated their efficiency in the design of TLR4 modulators with anionic amphiphilic structures.[29,30] Structural modifications have been projected by varying the number, the nature, and the length of the lipid chains and the number and disposition of amino groups in order to evaluate how these structural elements influence the TLR4 activity.

Synthesis of Glucose-Derived Cationic Glycolipids. The syntheses of the tri-*O*-alkylated 6-amino-6-deoxyglucoside derivatives **1** and **2** (Scheme 1) were successfully accomplished

Scheme 1. Syntheses of Monosaccharides 1−4[a]



[a]Reagents and conditions: (a) 1-bromohexane, NaH, DMF, overnight, 48% or 1-bromotetradecane, NaH, DMF, 55 °C, 52%; (b) H_2, Pd/C, MeOH, 2 h, 87% or PPh_3, THF, then, NH_4OH, 50 °C, overnight, 82%; (c) 1M $LiAlH_4$ in THF, $AlCl_3$, DCM, Et_2O, 83%; (d) I_2, PPh_3, imidazole, toluene, 94%; (e) $HS(CH_2)_2NHBoc$, Cs_2CO_3, DMF, 60 °C, 99%; (f) 1:1 TFA-DCM, 80%; (g) $AlCl_3$, DCM, Et_2O, 87%; (h) I_2, PPh_3, imidazole, toluene, 91%; (i) $HS(CH_2)_2NHBoc$, Cs_2CO_3, DMF, 60 °C, 95%; (j) 1:1 TFA-CH_2Cl_2, quant.

by reaction of the known methyl 6-azido-6-deoxy-α-D-glucopyranoside **12**[31] with hexyl or tetradecyl bromide and sodium hydride (**13** and **14**), followed by reduction of the azido group by either catalytic hydrogenation or Staudinger reaction with triphenylphosphine and hydrolysis of the corresponding phosphazene intermediate.[32] The target ether-type amino glycolipids **1** and **2** were isolated as the corresponding hydrochloride salts.

The 2,3-di-*O*-hexyl and -*O*-tetradecyl glucose derivatives **3** and **4** were synthesized from the corresponding 4,6-*O*-(*p*-methoxybenzylidene) protected precursor **15** and **16**,[33] respectively, which at their turn were obtained by standard alkylation of methyl 4,6-*O*-(*p*-methoxybenzylidene)-α-D-glucopyranoside.[34] The regioselective opening of the acetal ring of **15** using lithium aluminum hydride ($LiAlH_4$) gave the 4-*O*-*p*-methoxybenzyl (PMB) ether **17**. Compound **16** on this side was completely deprotected on C-4 and C-6 positions using aluminum trichloride, affording compound **20**. Iodination of the C-6 hydroxyl groups of **17** and **20** using Garegg's conditions[35] afforded compounds **18** and **21**, which were subjected to cesium carbonate-promoted nucleophilic displacement with *t*-butoxycarbonyl (Boc)-protected cysteamine (**20** and **22**) and final Boc removal in acidic conditions to give compounds **3** and **4**.

The synthetic routes to obtain compounds **5** and **6**, having a tertiary and two primary amino groups in the cationic head (Scheme 2), are based on the thiourea-forming and the copper(I)-catalyzed azide−alkyne cycloaddition (CuAAC) reactions, two "click"-type ligation strategies already proven very efficient to generate polycationic clusters.[36] The preparation of **5** started by hexanoylation of methyl 6-deoxy-6-iodo-α-D-glucopyranoside **23**[35] (**24**; Scheme 2, 2a), followed by nucleophilic displacement of the iodine by Boc-protected cysteamine (**25**) and carbamate hydrolysis, afforded the cysteaminyl derivative **26** in 92% overall yield. Condensation of **26** with 2-[*N*,*N*-bis(2-(*N*-*tert*-butoxycarbonylamino)-ethylamino]ethyl isothiocyanate[37] and final acid-promoted Boc deprotection provided **5**, which was isolated as the dihydrochloride salt.

The cationic amphiphile **6** was obtained in good yield from azide **12** by following a divergent synthetic strategy in which the hydrophobic and cationic domains are sequentially installed onto the glucopyranoside scaffold. Acylation of **12** with hexanoic anhydride and *N*,*N*-dimethylaminopyridine (DMAP) in DMF afforded triester **28** (Scheme 2, 2b) that was reacted with 3-bis[2-(*tert*-butoxycarbonylamino)ethyl]-propargylamine[38] in the presence of silica-based particles incorporating bis(pyridyl)amine (BPA) Cu(I) chelating agent[39] to give the triazol adduct **29** in 78% yield. The use of the solid-supported catalyst has proven advantageous even in multi-CuAAC ligation strategies, highly simplifying the purification step to a simple filtration process.[40] Acid hydrolysis of the Boc protecting groups in **29** provided the corresponding triamine **30**, which was next reacted with 2-(*N*-*tert*-butoxycarbonylamino)ethyl isothiocyanate[41] to give bis-(thiourea) **31**. Final hydrolysis of the Boc protecting groups led to the target compound **6**.

Syntheses of Trehalose-Derived Cationic Glycolipids. The strategies implemented for the preparation of the α,α′-trehalose amino glycolipids **7**−**11** parallel those above commented for the corresponding ether- (**1**−**4**) or ester-type (**5**−**7**) methyl α-D-glucopranoside counterparts. Thus, compounds **7** and **8** were obtained in good overall yield in an efficient five-step synthesis starting from 6,6′-di-*O*-trityl-α,α′-trehalose **32**[42] after alkylation of the six secondary hydroxyl groups (**33** and **34**), trityl cleavage with *p*-toluenesulfonic acid in DCM-MeOH (**35** and **36**), Garegg's iodination of the primary hydroxyls (**37** and **38**), nucleophilic displacement of the iodines with Boc-protected cysteamine (**39** and **40**), and hydrolysis of the carbamate groups (Scheme 3). The hexanoylated analogue **9** was similarly obtained from 6,6′-dideoxy-6,6′-diiodo-α,α′-trehalose **41**[43] by esterification of the secondary hydroxyls (**42**), incorporation of the Boc-protected

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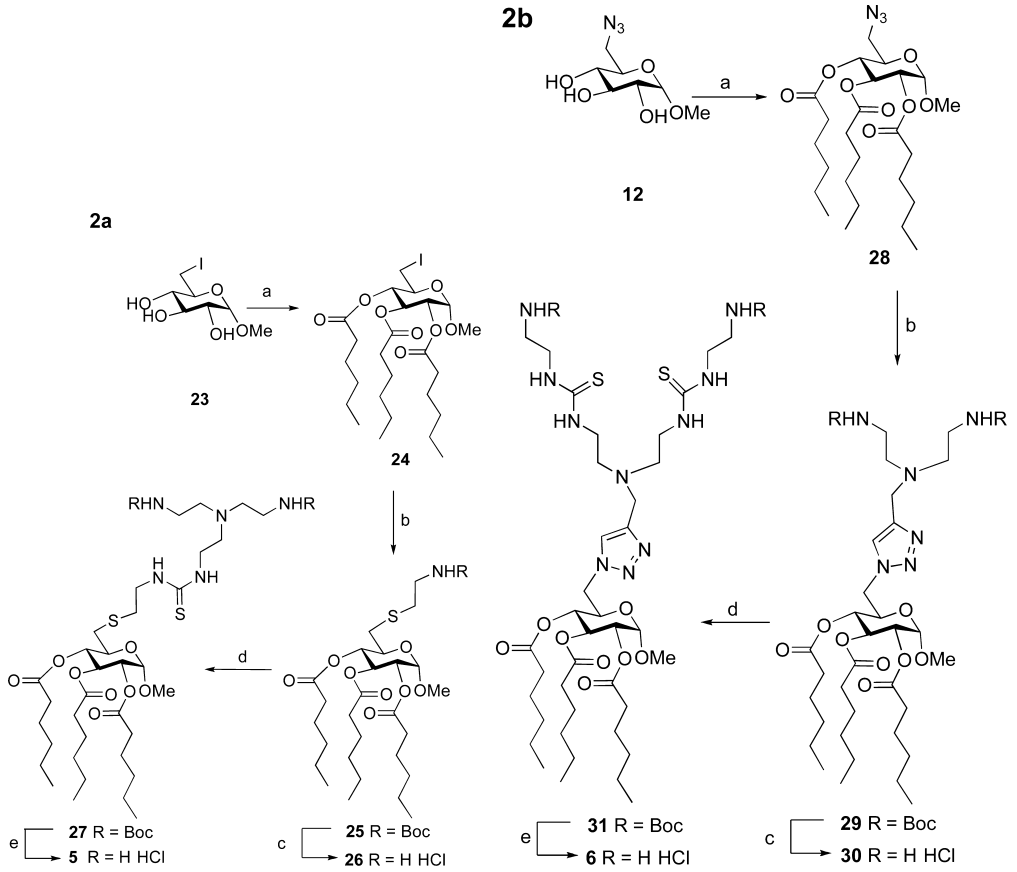
Scheme 2. Syntheses of Monosaccharides 5 (2a) and 6 (2b)[a]



[a]Reagents and conditions (2a): (a) hexanoic anhydride, DMAP, DMF, Ar, rt, 4 h, 55%; (b) *tert*-butyl *N*-(2-mercaptoethyl)carbamate, Cs_2CO_3, DMF, Ar, overnight, 85%; (c) 1:1 TFA-DCM, quant; (d) 2-[*N*,*N*-bis(2-(*N*-*tert*-butoxyaminocarbonyl)ethylamino]ethyl isothiocyanate, Et_3N, DCM, Ar, overnight, 50%; (e) 1:1 TFA-DCM, rt, 1 h, quant. Reagents and conditions (2b): (a) hexanoic anhydride, DMAP, DMF, Ar, 4 h, 55%; (b) 3-bis[2-(*tert*-butoxycarbonylamino)ethyl]propargylamine, Si-BPA·Cu^+, 9:1 H_2O/^{t}BuOH, 85 °C, 36 h. 78%; (c) 1:1 TFA/H_2O, rt, 1 h, quant; (d) 2-(*N*-*tert*-butoxyaminocarbonyl)ethyl isothiocyanate, Et_3N, DCM, overnight, 52%; (e) 1:1 TFA/H_2O, rt, 1 h, quant.

cysteamine substituents at the primary positions (**43**), and final deprotection (Scheme 4).

The cationic trehalose amphiphiles **10** and **11** were prepared starting from the common diazide precursor **44**, readily accessed by nucleophilic displacement of the iodine in **42** with sodium azide by CuAAC ligation using the silica-supported Si-BPA-Cu^+ catalyst. Thus, sequential reaction of **44** with *N*-Boc-propargylamine (**45**), carbamate hydrolysis (**46**), thiourea-coupling with Boc-protected 2-aminoethyl isothiocyanate (**47**), and final Boc removal yielded the diaminoethylthioureido adduct **10**. Alternatively, the CuAAC coupling of **44** with 3-bis[2-(*tert*-butoxycarbonylamino)ethyl]-propargylamine (**48**) followed by carbamate hydrolysis afforded compound **11** (Scheme 5).

TLR4 Modulation in HEK-Blue Cells. Cationic amphiphiles **1**−**11** were first screened for their capacity to interfere with TLR4 activation and signaling on HEK-Blue cells. HEK-Blue cells are stably transfected with TLR4, MD-2, and CD14 genes. In addition, these cells stably express an optimized alkaline phosphatase gene engineered to be secreted (sAP), placed under the control of a promoter inducible by several transcription factors such as NF-κB and AP-1.[29] This reporter gene allows monitoring the activation of TLR4 signal pathway by endotoxin. Compounds **5** and **9**−**11** were inactive in stimulating TLR4 signal when provided alone while inhibited in a dose-dependent way the LPS-stimulated TLR4 signal (Table 1). Compounds **1**−**4** and **6**−**8** resulted as being inactive or with very low activity both as agonists and antagonists.

The lack of significant activity of all compounds bearing C_6 or C_{14} ether-linked lipophilic chains, namely compounds **1**−**4** and **7**−**8**, strongly suggests that the presence of ester-type linkages at the hydrophobic domain is a primary structural requirement to elicit LPS-antagonist behavior in cationic glycolipids. From the acylated sublibrary, all compounds are active with the exception of compound **6**, meaning that the cationic head also has an impact on the TLR4 antagonist activity. Among these, the trehalose-based disaccharides **9**−**11**, bearing more compact cationic headgroups, showed higher potency as TLR4 antagonists than monosaccharide **5**. The observed trend points to a positive relationship between well-ordered facial amphiphilicity and TLR4 antagonist activity of cationic glycolipids. In agreement with this, disaccharide **11**, with six hexanoyl chains and six protonable amino groups oriented toward opposite faces in a rather compact arrangement (Figure 3), proved to be the most active TLR4 antagonist. Compounds **5**, **9**, **10**, and **11** were further tested for their toxicity by a standard MTT viability test, and all resulted in being nontoxic or with very low toxicity in the concentration range used to test their activity (Supporting Information).

Activity on HEK293 Cells Transfected with Human and Murine MD-2·TLR4. Biologically active cationic glyco-

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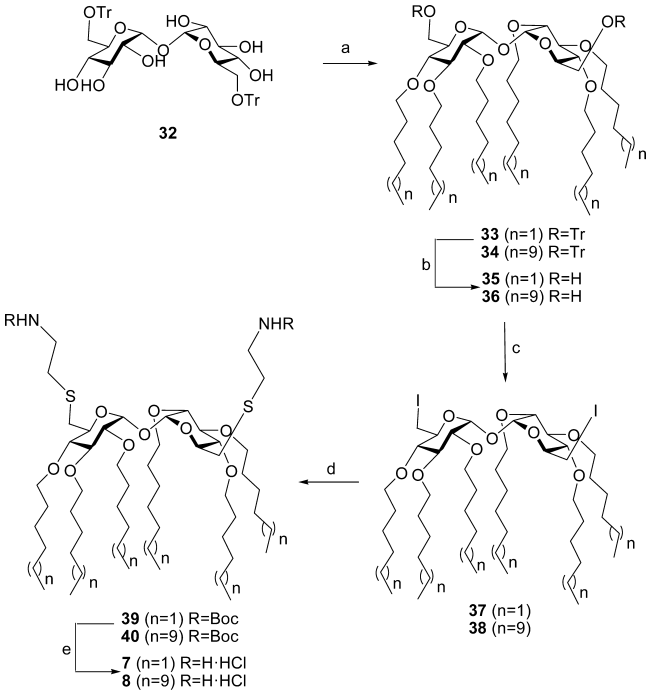
Scheme 3. Syntheses of Trehalose Derivatives 7 and 8[a]



[a]Reagents and conditions: (a) hexyl bromide, NaH, DMF, overnight, 92% or 1-bromotetradecane, NaH, DMF, 50 °C, 77%; (b) *p*-toluenesulfonic acid, 1:1 DCM−MeOH, rt, 4 h, 48% for **35** and 47% for **36**); (c) I_2, PPh_3, imidazole, toluene, 70 °C, 3 h, 94% for **37** and 96% for **38**; (d) $HS(CH_2)_2NHBoc$, Cs_2CO_3, DMF, 60 °C, overnight, 85% for **39** and 99% for **40**; (e) 1:1 TFA/DCM, rt, 15 min, quant for **7**, 98% for **8**.

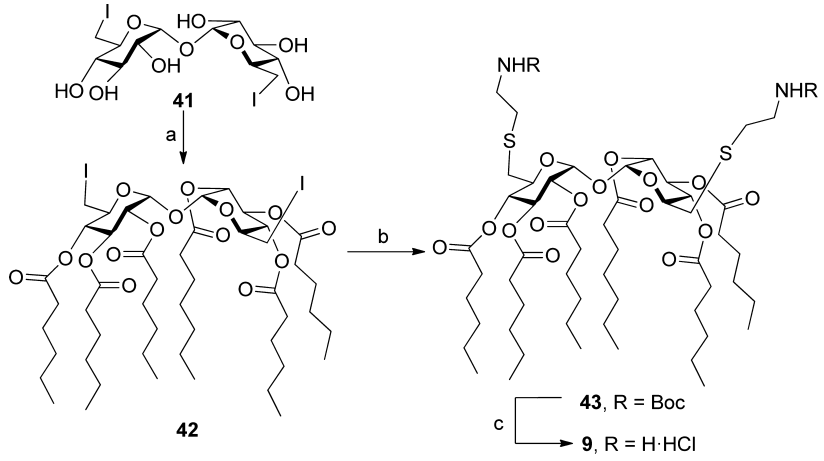
lipids **5**, **9**, **10**, and **11** were further examined for their capacity to stimulate or to inhibit LPS-induced TLR4 activation and signaling in HEK293 cells transfected with human or murine TLR4 and MD-2 and a dual luciferase reporter gene (Figure 4).

In the absence of LPS, none of the cationic glycolipids stimulate TLR4 signaling (no agonist activity) in cells transfected with hMD-2·hTLR4 or mMD-2·mTLR4. Conversely, in the presence of LPS, all compounds were able to inhibit human and murine MD-2·TLR4 activation in a concentration-dependent manner. The antagonist potency (IC_{50} values, Table 1) of **5**, **9**, **10**, and **11** was similar in cells transfected with human and mouse receptors and also very similar to the activity found in HEK-Blue cells (Table 1). In cells transfected with hMD-2·hTLR4 or mMD-2·mTLR4, triacylated monosaccharide **5** and hexacylated disaccharide **9** were less active (IC_{50} = 3.3−3.9 and 0.8−1.4 μM, respectively) than disaccharides **10** and **11** (IC_{50} = 0.6 and 0.2 μM, respectively). The high potency of trehalose-derived glycolipids **9** and **11** in inhibiting both mouse and human MD-2·TLR4 signals is reminiscent of the activity of synthetic Eritoran that has potent TLR4 antagonist activity in all species.[16,44] In contrast, natural lipid IVa is agonist on murine and antagonist on human TLR4.[45]

Experiments on Murine Macrophages. The activity of compounds **5**, **9**, and **11** on LPS-induced TLR4 signaling in bone marrow-derived murine macrophages (BMDM) was subsequently tested. Compounds **5** and **9** showed very low/no activity in activating TLR4 or in inhibiting LPS-stimulated TLR4 signal (Supporting Information), while compound **11** (Figure 5) gave a concentration-dependent inhibition of IL-6 and TNF-α production at concentrations of 1 and 2 μM (Figure 5), while at 0.1 and 0.5 μM, concentrations had no effect or a slightly potentiating effect.

Aggregation Properties of Cationic Glycolipids. Cationic lipids can spontaneously assembly into liposomal structures. Some cationic liposomes induce the expression of pro-apoptotic and pro-inflammatory mediators through the activation of cellular pathways.[17] However, it is still controversial if the initiation of inflammatory and apoptotic response is due to specific interaction with receptors at the cell surface or to the internalization of liposomes into cells through endocytosis and endocytosis-like mechanisms followed by interaction of charged compounds with downstream effectors. In the particular case of cationic lipids modulating TLR4 activity, there is no information available on whether aggregated species or single molecules are the active species. To have an insight in this question, we have determined the critical micelle concentrations (CMC) of cationic glycolipids **5**, **9**, **10**, and **11** using an established technique based on the polarity-induced change in the fluorescence spectra of pyrene when incorporated in micelles formed by synthetic compounds (Table 2).[46] In all

Scheme 4. Synthesis of Trehalose Derivative 9[a]



[a]Reagents and conditions: (a) $HS(CH_2)_2NHBoc$, Cs_2CO_3, DMF, 60 °C, 24 h, 58%; (b) 1:1 TFA/DCM, rt, 15 min, quant.

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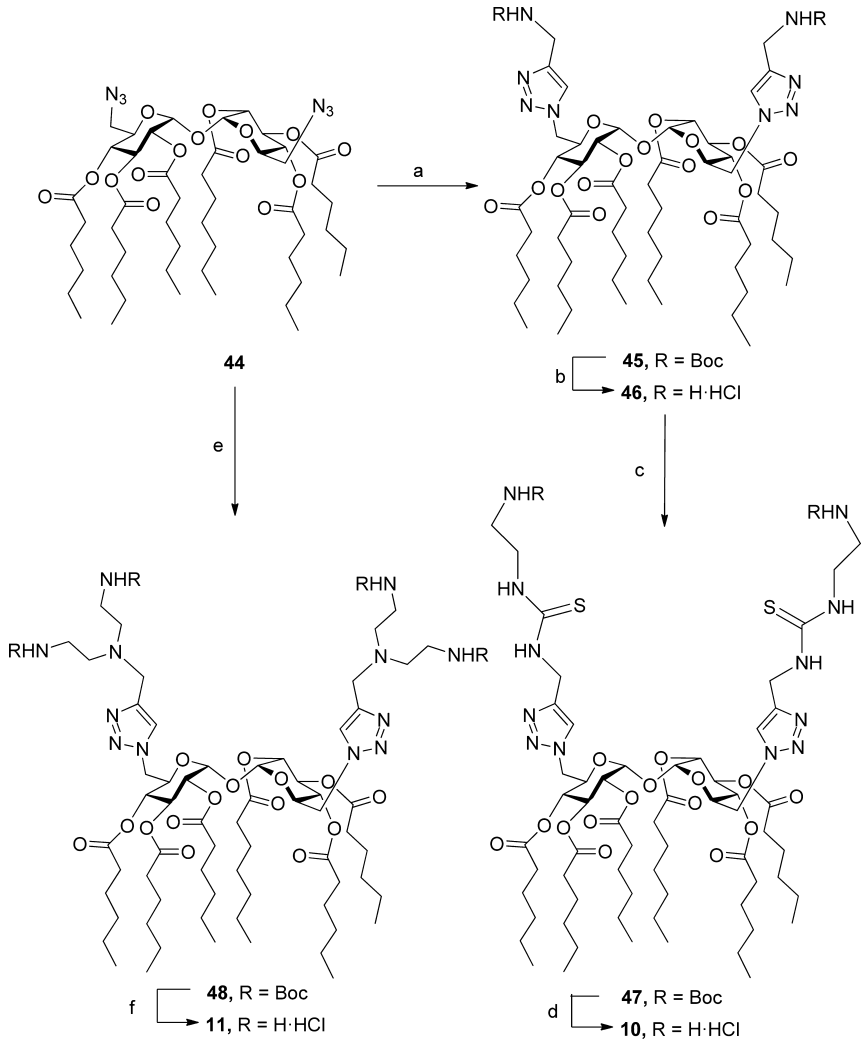
Scheme 5. Syntheses of Trehalose Derivatives 10 and 11[a]



[a]Reagents and conditions: (a) *N*-*tert*-butoxycarbonylpropargylamine, Si-BPA·Cu^+, 9:1 H_2O/^{t}BuOH, 24 °C, 36 h, quant; (b) 1:1 TFA/H_2O, rt, 1 h, 97%; (c) *tert*-butyl-*N*-(2-isothiocyanoethyl)carbamate, Et_3N, DCM, overnight, 67%; (d) 1:1 TFA/H_2O, rt, 1 h, quant; (e) 3-bis[2-(*tert*-butoxycarbonylamino)ethyl]propargylamine, Si-BPA·Cu^+, 9:1 H_2O/^{t}BuOH, reflux, 36 h, 91%; (f) 1:1 TFA/H_2O, rt, 1 h, quant.

Table 1. TLR4 Antagonist Activity of Cationic Glycolipids 5 and 9−11 on HEK-Blue Cells, HEK293 hMD-2/hTLR4, and HEK293 mMD-2/mTLR4 Stimulated with *E. coli* O55:B5 LPS (10 nM)

	IC_{50} (μM)		
compd	HEK-Blue	HEK293 hMD-2·hTLR4	HEK293 mMD-2·mTLR4
5	3.7 ± 0.4	3.9 ± 1.5	3.3 ± 1.2
9	1.3 ± 0.1	1.4 ± 0.3	0.8 ± 0.2
10	5.0 ± 1.0	0.6 ± 0.02	0.6 ± 0.03
11	0.6 ± 0.05	0.2 ± 0.02	0.2 ± 0.03

cases, the CMC values of active compounds are higher than the corresponding IC_{50} values as TLR4 antagonists, suggesting that the biologically active species are prevalently single molecules in solution.

Synthesis and Biological Activity of Gold Nanoparticles Coated with Glycolipid 11. LPS is an amphiphilic molecule, and it is mainly present in the form of micellar aggregates in a concentration range that is relevant for its biological activity. It has been recently proposed that the multiple presentation of LPS or other TLR4 ligands on nanoparticles could be a way to potentiate the agonist or antagonist action of chemicals by mimicking the 3D-structure of LPS aggregates.[47,48] Moreover, the possibility of in vitro and/or in vivo delivery based on NP is considered advantageous for clinical development, as it can maximize the effectiveness of drugs, minimize the invasiveness and toxic side effects, and speed up the clinical development program. To test the suitability of this approach in the case of cationic glycolipids, the preparation and biological evaluation of gold nanoparticles coated with the most active compound **11** has been undertaken. Colloidal gold nanoparticles were synthesized by a variation of the Brust−Schiffrin method[49] and coated by surface adsorption with the α,α′-trehalose derivative **11**. The resulting cationic glycolipid-modified nanoparticles (**11**-NP)

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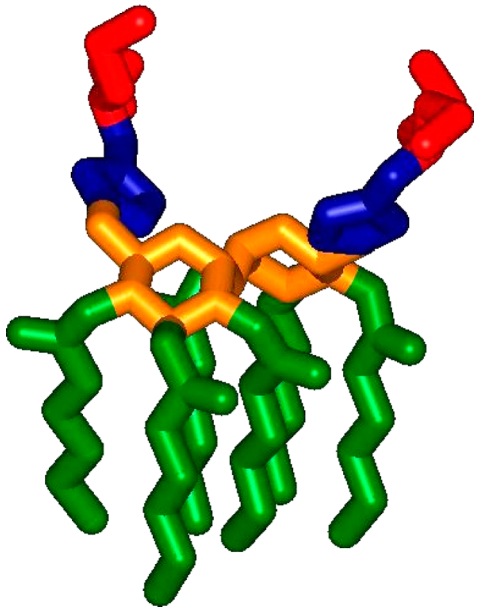

Figure 3. 3D molecular model of compound **11** (cationic headgroups in red, triazol linkers in blue, α,α'-trehalose scaffold in orange, hexanoyl chains in green) evidencing its compact facial amphiphilic character. Hydrogens have been omitted for the sake of clarity.

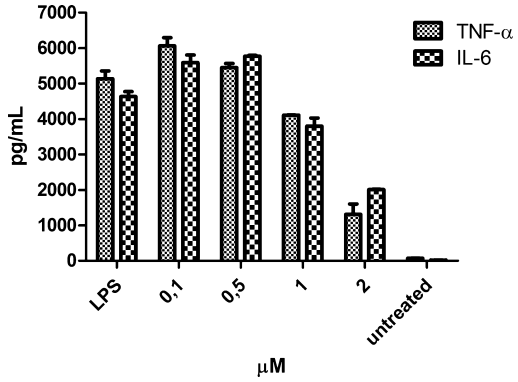

Figure 5. BMDM were treated with increasing concentrations (0−2 μM) of compound **11** in RPMI + FBS 10% in the presence of LPS, administered 1 h after the treatment with synthetic compound. The ELISA assay, performed after overnight incubation, revealed a dose dependent decrease of LPS-induced IL-6 and TNF-α production. Cytokines productions in cells not treated with LPS are reported as negative controls.

Table 2. Critical Micelle Concentrations (CMC) in Aqueous Environment of TLR4 Antagonists

compd	CMC (μM)
5	59.7 ± 7.4
9	97.7 ± 10.0
10	10.9 ± 2.1
11	350.5 ± 70.5

kept a small size with low polydispersity. Most interestingly, they retained the biological activity without apparent increase in cytotoxicity. These results represent a proof-of-concept of the possibility of developing nanoparticulate systems based on cationic glycolipids as modulators of TLR4 signaling pathway, an approach previously demonstrated only for LPS itself.[47] On the basis of these data, the prepared **11**-NP nanoparticles were

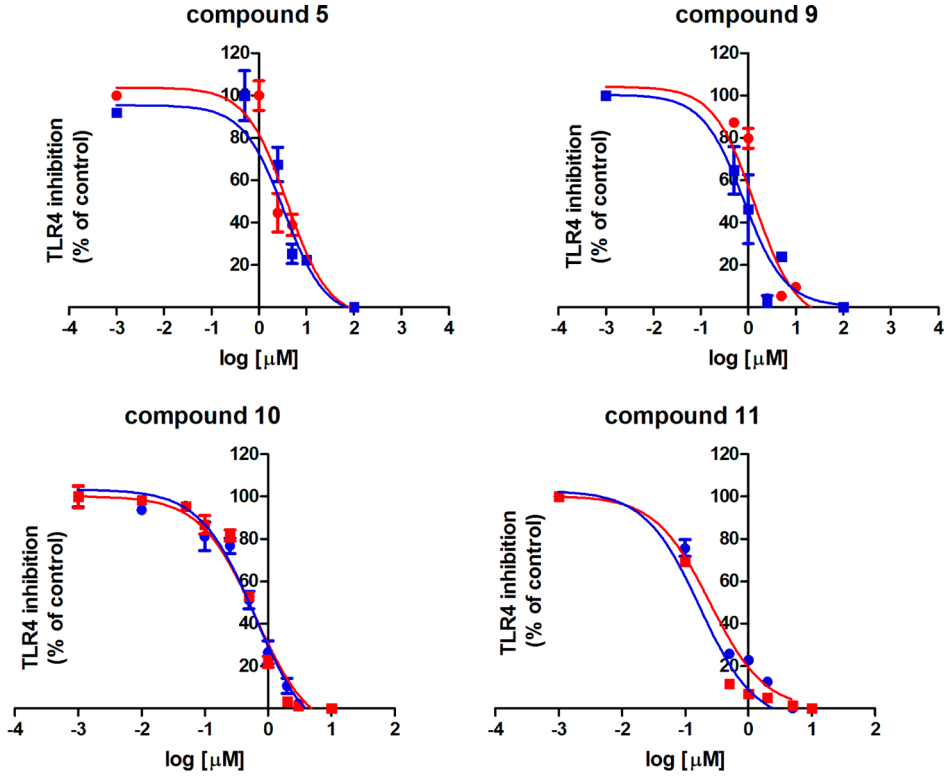

Figure 4. Dose-dependent inhibition of LPS-stimulated TLR4 activation by synthetic glycolipids. HEK293 cells transfected with human MD-2·TLR4 (red line) or murine MD-2/TLR4 (blue line) were treated with increasing concentrations of compounds and stimulated with LPS (5 nM). Normalized data are representative of three independent experiments.

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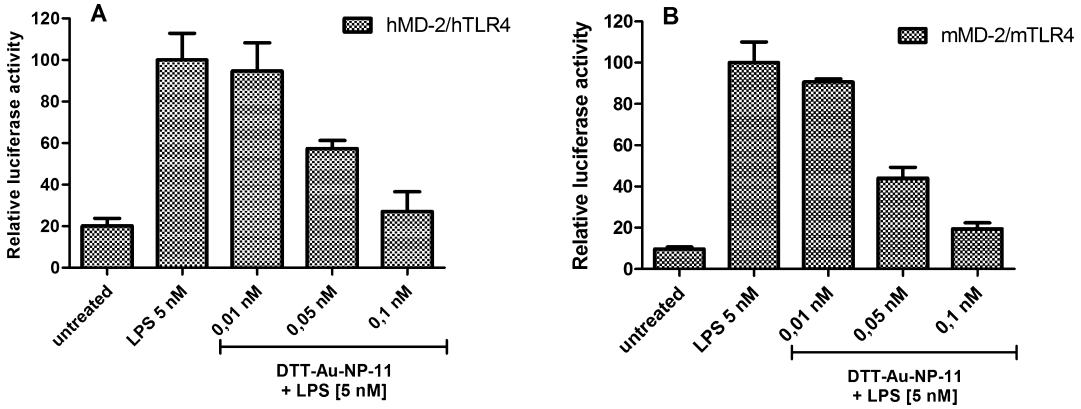

Figure 6. Dose-dependent TLR4 antagonism in HEK293 cells treated with DTT-Au-NP-**11**. HEK293 cells were transfected with NF-κB-dependent luciferase and constitutive Renilla luciferase reporter plasmids as well as with (A) human or (B) murine MD-2 and TLR4 plasmids. The indicated amount of the DTT-Au-NP-**11** was added to the cells, followed 1 h later by stimulation with LPS. Luciferase activity was measured 16 h later.

engaged in an in vivo assay. The inhibitory activity of the nanoparticles coated with glycolipid **11** was tested on HEK293 cells. They exhibited strong LPS inhibitory activity already at very low concentrations (Figure 6A,B) on the human as well as the murine MD-2·TLR4 receptor complex.

In Vivo Activity. Because the synthesized compounds exhibited strong inhibition of the LPS-induced MD-2·TLR4 activation in vitro, we next wished to determine their inhibitory potential in vivo. All four selected candidates (**5**, **9**, **10**, and **11**) potently inhibited the LPS-induced immune activation in C57/Bl6 mice (Figure 7). The strongest inhibition was exhibited by

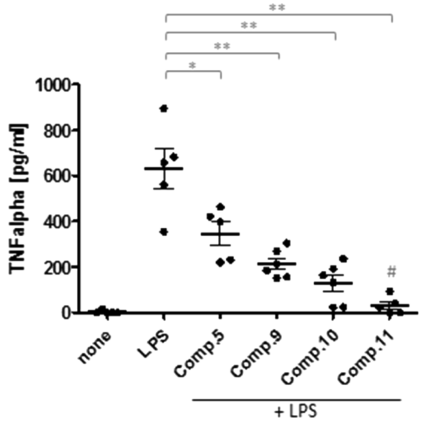

Figure 7. In vivo activity of cationic amphiphiles. C57/Bl6 mice were injected ip with the indicated compounds (2×10^{-7} mol/mouse), followed 1 h later by ip injection of LPS (1×10^{-9} mol/mouse). Three hours later, sera were collected and TNF-α concentration was determined by ELISA (data shown with mean and standard error, $N = 5-6$) two-tailed t test (* $p < 0.1$; ** $p < 0.01$—compared to ≫LPS≪) (# not significant—compared to ≫none≪).

compound **11**, which totally abolished LPS-induced immune activation. Results in Figure 7 show that compounds **5**, **9**, **10**, and **11** are strong MD-2·TLR4 inhibitors not only in vitro but also in vivo.

CONCLUSIONS AND PERSPECTIVES

New cationic amphiphiles **1**—**11** based on monosaccharide and disaccharide glycolipid scaffolds have been designed, synthesized, and their capacity to modulate TLR4 activation and signaling evaluated. Glucose-based compound **5** and trehalose-based compounds **9**—**11** were active in inhibiting the LPS-triggered TLR4 activation and signaling in HEK cells, with IC_{50} values ranging from about 5 to 0.2 μM. The cell toxicity of these molecules is low, and the potency of TLR4 antagonism is in the same order of magnitude of the best synthetic TLR4 antagonists so far tested by us[29] and other groups.[30] The active molecules inhibited the TLR4 signal in HEK cells transfected with human and murine MD-2·TLR4 complexes with very similar potency, similarly to the very efficient TLR4 antagonist Eritoran and differently from the natural TLR4 antagonist lipid IVa that has species-specific activity (antagonist on human and agonist on mice MD-2·TLR4). Compounds **5**, **9**, **10**, and **11** significantly inhibited LPS-triggered IL-6 production in mice, with compound **11** showing the most evident effect. Because these compounds are active in vitro and in vivo and show low toxicity, they represent good leads for the development of drugs targeting TLR4 signaling.

The biological evaluation of active compounds compared to inactive, structurally related monosaccharides (compounds **1**—**4** and **6**) and disaccharides (compounds **7** and **8**) suggests some general structure—activity relationships in this type of compounds: (i) the presence of acyl lipophilic chains at the hydrophobic domain seems to be a primary requisite because all compounds with ether bonds are inactive, and (ii) the higher in vitro and in vivo activity of compound **11** suggests that the trehalose scaffold favors the biological activity, probably by providing a well-ordered facial amphiphilic character. Compound **11** adsorbed on gold nanoparticles (**11**-NP) is still active as TLR4 antagonist in cells, but the high toxicity of these functionalized nanoparticles could prevent their use in vivo as carriers for TLR4 antagonists.

Finally, the experimentally determined CMC values for cationic glycolipids **5** and **9**—**11** are 1 order of magnitude higher than the corresponding IC_{50} as TLR4 antagonists, suggesting that they are active as single monomers in solution. This very likely means that specific molecular interaction with CD14 and MD-2 receptors regulate the TLR4 activity of these compounds. It will be important in the future to define more precisely the molecular determinants of the interaction with CD14 and MD-2 receptors to allow a structure-based rational design of cationic TLR4 modulators.

EXPERIMENTAL SECTION

General Synthetic Methods. Optical rotations were measured at 20 ± 2 °C in 1 dm tubes on a Jasco P-2000 polarimeter. Ultraviolet—

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visible (UV) spectra were recorded in 1 cm tubes on a Jasco V-630 spectrophotometer. Infrared (IR) spectra were recorded on a Jasco ATR MIRacle spectrophotometer. ^{1}H (and ^{13}C NMR) spectra were recorded at 300 (75.5) and 500 (125.7) MHz with Bruker 300 AMX, 500 AMX, and 500 DRX. 1D TOCSY, 2D COSY, HMQC, and HSQC experiments were used to assist on NMR assignments. Thin-layer chromatography (TLC) was carried out on aluminum sheets coated with Sílica Gel 60 F_{254} Merck with visualization by UV light and by charring with 10% H_2SO_4. With preparative purposes, column chromatography was carried out on Silica Gel 60 F_{254} Merck. ESI mass spectra were recorded in the positive mode on a Bruker Daltonics esquire6000 ion-trap mass spectrometer. Typically, samples were dissolved in appropriate volumes of deionized water to give samples concentration of 50 mg/L. Aliquots were mixed with 25:25:1 deionized water−methanol−trifluoroacetic acid, generally in a ratio of 1:10, to give a total volume of 200 μL. Samples were introduced by direct infusion, using a Cole−Parmer syringe at a flow rate of 2 μL/min. Ions were scanned between 300 and 3000 Da with a scan speed of 13000 Da/s at unit resolution using resonance ejection at the multipole resonance of one-third of the radio frequency (Ω = 781.25 kHz). Calibration of the mass spectrometer was performed using ES tuning mix (Hewlett-Packard). Recorded data were processed using Bruker Daltonics Esquire 5.0 software (Bruker). Elemental analyses were carried out at the Instituto de Investigaciones Químicas (Sevilla, Spain) using an elemental analyzer Leco CHNS-932 or Leco TruSpec CHN.

Methyl 6-azido-6-deoxy-α-D-glucopyranoside,[28] methyl 4,6-*O*-(4-methoxybenzylidene)-α-D-glucopyranoside,[34] methyl 6-deoxy-6-iodo-α-D-glucopyranoside,[35] 2-[*N*,*N*-bis(2-(*N*-*tert*-butoxyaminocarbonyl)-ethylamino]ethyl isothiocyanate,[37] 6,6′-dideoxy-6,6′-diiodo-α,α′-trehalose **41**,[43] 6,6′-di-*O*-trityl-α,α′-trehalose **32**,[42] *N*-(2-isothiocyanoethyl) *tert*-butylcarbamate,[41] and 3-bis[2-*tert*-butoxycarbonylamino)ethyl]-propargylamine[38] were obtained according to described procedures. Purity of all final compounds was confirmed to be ≥95% by ^{1}H NMR and combustion microanalysis.

*Methyl 6-Azido-6-deoxy-2,3,4-tri-O-hexyl-α-D-glucopyranoside (**13**).* To a solution of methyl 6-azido-6-deoxy-α-D-glucopyranoside (0.40 m, 1.82 mmol) in dry DMF (9 mL), NaH (0.65 g, 16.42 mmol) was added, under Ar atmosphere, at 0 °C. Then 1-bromohexane (2.3 mL, 16.42 mmol) was added dropwise, and the reaction mixture was stirred overnight at rt. The solvent was evaporated and the residue diluted in DCM (10 mL) and washed with H_2O (2 × 10 mL). The organic layer was dried ($MgSO_4$), concentrated, and purified by column chromatography (1:30 EtOAc−cyclohexane). Yield 48% (0.50 m, 1.06 mmol); R_f = 0.34 (1:18 EtOAc−cyclohexane); $[\alpha]_D$ = +90.0 (*c* 1.0, DCM). IR: ν_{max} = 2099, 1094 cm^{-1}. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 4.74 (d, 1 H, $J_{1,2}$ = 3.5 Hz, H-1), 3.79, 3.64, 3.58 (m, 6 H, OCH_2), 3.67 (m, 1 H, H-5), 3.64−3.24 (m, 2 H, H-3, H-6a), 3.39 (s, 3 H, OCH_3), 3.35 (m, 1 H, H-6b), 3.23 (dd, 1 H, $J_{2,3}$ = 9.6 Hz, H-2), 3.12 (t, 1 H, $J_{3,4}$ = $J_{4,5}$ = 9.3 Hz, H-4), 1.55 (m, 6 H, CH_2), 1.27 (m, 18 H, CH_2), 0.86 (m, 9 H, CH_3). ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 98.0 (C-1), 81.3 (C-3), 80.7 (C-2), 78.8 (C-4), 73.7, 73.6, 71.8 (OCH_2), 70.2 (C-5), 55.2 (OCH_3), 51.5 (C-6), 31.7−22.6 (CH_2), 14.3 (CH_3). ESI MS: *m*/*z* = 965.8 [2 M + Na]$^+$, 494.6 [M + Na]$^+$. Anal. Calcd for $C_{25}H_{49}N_3O_5$: C, 63.66; H, 10.47; N, 8.91. Found: C, 63.72; H, 10.97; N, 8.33.

*Methyl 6-Amino-6-deoxy-2,3,4-tri-O-hexyl-α-D-glucopyranoside Hydrochloride (**1**).* To a solution of **13** (0.15 g, 0.32 mmol) in degassed MeOH (12 mL), Pd/C (10%, 0.06 g) was added and the mixture was stirred under H_2 atmosphere (1 bar) at rt until complete consumption of the starting compound. The catalyst was filtered off, the solution concentrated, and the resulting residue purified by column chromatography (1:9 EtOAc−cyclohexane → 45:5:3 EtOAc−EtOH−H_2O) and freeze-dried from 0.1 N HCl solution. Yield 87% (0.12 g, 0.26 mmol); $[\alpha]_D$ = +83.0 (*c* 1.0, DCM). ^{1}H NMR (300 MHz, CD_3OD): δ = 4.82 (d, 1 H, $J_{1,2}$ = 3.4 Hz, H-1), 3.81, 3.64, 3.56 (m, 6 H, OCH_2), 3.65 (m, 1 H, H-5), 3.53 (t, 1 H, $J_{2,3}$ = $J_{3,4}$ = 9.3 Hz, H-3), 3.41 (s, 3 H, OCH_3), 3.25 (dd, 1 H, H-2), 3.11 (dd, 1 H, $J_{6a,6b}$ = 13.1 Hz, $J_{5,6a}$ = 2.7 Hz, H-6a), 3.01 (t, 1 H, $J_{4,5}$ = 9.3 Hz, H-4), 2.84 (dd, 1 H, $J_{5,6b}$ = 8.5 Hz, H-6b), 1.56 (m, 6 H, CH_2), 1.33 (m, 18 H, CH_2), 0.91 (m, 9 H, CH_3). ^{13}C NMR (75.5 MHz, CD_3OD): δ = 99.1 (C-1), 82.6 (C-3), 81.8 (C-2), 81.1 (C-4), 74.5, 74.3, 72.2 (OCH_2), 70.7 (C-5), 55.8 (OCH_3), 42.9 (C-6), 33.0−24.2 (CH_2), 14.4 (CH_3). ESIMS: *m*/*z* = 891.7 [2 M + H]$^+$, 446.5 [M + H]$^+$. Anal. Calcd for $C_{25}H_{51}NO_5$·HCl: C, 62.28; H, 10.87; N, 2.91. Found: C, 62.33; H, 10.69; N, 2.70.

*Methyl 6-Azido-6-deoxy-2,3,4-tri-O-tetradecyl-α-D-glucopyranoside (**14**).* To a solution of methyl 6-azido-6-deoxy-α-D-glucopyranoside (0.28 g, 1.30 mmol) in dry DMF (4 mL), NaH (0.55 mg, 13.60 mmol) was added, under Ar atmosphere, at 0 °C. Then 1-bromotetradecane (4.1 mL, 13.60 mmol) was added dropwise, and the reaction mixture was stirred overnight at 55 °C. The solvent was evaporated and the residue diluted in DCM (10 mL) and washed with H_2O (2 × 10 mL). The organic layer was dried ($MgSO_4$), concentrated, and purified by column chromatography (cyclohexane → 1:40 EtOAc−cyclohexane). Yield 42% (0.45 g, 0.26 mmol); R_f = 0.25 (1:15 EtOAc−cyclohexane); $[\alpha]_D$ = +52.1 (*c* 1.0, DCM). IR: ν_{max} = 2100, 1096 cm^{-1}. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 4.76 (d, 1 H, $J_{1,2}$ = 3.4 Hz, H-1), 3.79, 3.64, 3,58 (m, 6 H, OCH_2), 3.69 (m, 1 H, H-5), 3.66−3.44 (m, 2 H, H-3, H-6a), 3.39 (s, 3 H, OCH_3), 3.37 (dd, 1 H, $J_{6a,6b}$ = 13.3 Hz, $J_{5,6b}$ = 5.5 Hz, H-6b), 3.25 (dd, 1 H, $J_{2,3}$ = 9.4 Hz, H-2), 3.13 (t, 1 H, $J_{3,4}$ = $J_{4,5}$ = 9.4 Hz, H-4), 1.57 (m, 6 H, CH_2), 1.27 (m, 66 H, CH_2), 0.86 (m, 9 H, CH_3). ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 98.0 (C-1), 81.3 (C-3), 80.7 (C-2), 78.8 (C-4), 73.7, 73.6, 71.8 (OCH_2), 70.2 (C-5), 55.2 (OCH_3), 51.5 (C-6), 31.9−22.7 (CH_2), 14.1 (CH_3). ESI MS: *m*/*z* = 830.8 [M + Na]$^+$. Anal. Calcd for $C_{49}H_{97}N_3O_5$: C, 72.81; H, 12.10; N, 5.20. Found: C, 72.89; H, 11.87; N, 4.88.

*Methyl 6-Amino-6-deoxy-2,3,4-tri-O-tetradecyl-α-D-glucopyranoside Hydrochloride (**2**).* To a solution of **14** (0.15 g, 0.19 mmol) in THF (16.4 mL), TPP (0.10 g, 0.39 mmol) was added and the mixture was stirred at rt for 15 min. Then NH_4OH (1.6 mL) was added, the solution was stirred overnight at 50 °C, and then concentrated. The resulting residue was purified by column chromatography (EtOAc → 9:1 DCM−MeOH) and freeze-dried from 10:1 H_2O−HCl 0.1 N solution. Yield 82% (0.13 mg, 0.16 mmol); $[\alpha]_D$ = +43.2 (*c* 1.0, DCM). IR: ν_{max} = 1092 cm^{-1}. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 4.79 (d, 1 H, $J_{1,2}$ = 3.4 Hz, H-1), 3.78, 3.60, 3.55 (m, 7 H, OCH_2, H-3), 3.73 (m, 1 H, H-5), 3.46 (s, 3 H, OCH_3), 3.27 (m, 1 H, H-6a), 3.22 (dd, 1 H, $J_{1,2}$ = 9.5 Hz, H-2), 2.95 (m, 2 H, H-4, H-6b), 1.55 (m, 6 H, CH_2), 1.25 (m, 66 H, CH_2), 0.87 (t, 9 H, $^{3}J_{H,H}$ = 6.6 Hz, CH_3). ^{13}C NMR (75.5 MHz, CD_3OD): δ = 98.0 (C-1), 81.0 (C-3), 80.4 (C-2), 79.9 (C-4), 77.2 (C-5), 73.7, 73.5, 71.8 (OCH_2), 67.1 (C-6), 55.9 (OCH_3), 31.9−22.7 (CH_2), 14.0 (CH_3). ESI MS: *m*/*z* = 783.0 [M + H]$^+$. Anal. Calcd for $C_{49}H_{99}NO_5$·HCl: C, 71.88; H, 12.31; N, 1.71. Found: C, 71.64; H, 12.26; N, 1.49.

*Methyl 4,6-O-(4-Methoxybenzylidene)-2,3-di-O-hexyl-α-D-glucopyranoside (**15**).* To a solution of methyl 4,6-*O*-(4-methoxybenzylidene)-α-D-glucopyranoside (0.80 g, 2.57 mmol) in DMF (8 mL), NaH (0.62 g, 15.42 mmol) was added. Then, 1-bromohexane (1.8 mL, 12.85 mmol) was added dropwise, and the resulting mixture was stirred at 60 °C overnight. After cooling to rt, the reaction was quenched with MeOH (2 mL) and the solution was stirred for 20 min. Solvents were then evaporated, and the residue was diluted with EtOAc (50 mL) and citric acid (satd aq soln, 40 mL). The layers were separated, and the organic phase was washed with H_2O (3 × 40 mL), dried ($MgSO_4$), evaporated, and purified by column chromatography (1:9 EtOAc−cyclohexane). Yield 57% (0.71 g, 0.15 mmol); R_f = 0.44 (1:9 EtOAc−cyclohexane); $[\alpha]_D$ = +37.2 (*c* 1.0, DCM). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.39, 6.87 (2 d, 4 H, A_2X_2, aromatics), 5.49 (s, 1 H, PhC*H*), 4.78 (d, 1 H, $J_{1,2}$ = 3.8 Hz, H-1), 4.25 (dd, 1 H, $J_{6a,6b}$ = 9.6 Hz, $J_{5,6a}$ = 4.5 Hz, H-6a), 3.80 (s, 3 H, $PhOCH_3$), 3.76 (m, 1 H, H-5), 3.72 (t, 1 H, $J_{2,3}$ = $J_{3,4}$ = 9.2 Hz, H-3) 3.72−3.57 (m, 5 H, H-6b, OCH_2), 3.47 (t, 1 H, $J_{4,5}$ = 9.2 Hz, H-4), 3.42 (s, 3 H, OCH_3), 3.34 (dd, 1 H, H-2), 1.66−1.49 (m, 4 H, CH_2), 1.41−1.18 (m, 12 H, CH_2), 0.88, 0.84 (2 t, 6 H, $^{3}J_{H,H}$ = 6.5 Hz, CH_3). ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 159.9−113.5 (Ph), 101.2 (PhCH), 99.1 (C-1), 81.9 (C-4), 80.4 (C-2), 78.2 (C-3), 73.4, 72.2 (OCH_2), 69.0 (C-6), 62.4 (C-5), 55.6 (OCH_3), 31.7−22.6 (CH_2), 14.0 (CH_3). ESI MS: *m*/*z*: 519.5 [M

+ K]$^+$, 503.6 [M + Na]$^+$. Anal. Calcd for $C_{27}H_{44}O_7$: C, 67.47; H, 9.23. Found: C, 67.54; H, 9.30.

Methyl 4,6-O-(4-Methoxybenzylidene)-2,3-di-O-tetradecyl-α-D-glucopyranoside (16).[31] To a solution of methyl 4,6-O-(4-methoxybenzylidene)-α-D-glucopyranoside (0.80 g, 2.57 mmol) in DMF (8 mL), NaH (60% suspension in mineral oil, 0.62 g, 15.42 mmol) was carefully added in small portions. Tetradecyl bromide (3.8 mL, 12.85 mmol) was added dropwise and the resulting mixture was stirred at 60 °C overnight. After cooling to rt, the mixture was quenched with methanol (2 mL) then the solution was stirred for 20 min. Solvents were then evaporated and the residue was diluted with EtOAc (50 mL). Citric acid (satd aq soln, 40 mL) was added, the layers were separated, the organic layer was washed with water (3 × 40 mL), dried (Na_2SO_4) and evaporated. Flash column chromatography on silica gel of the residue (1:9 EtOAc−cyclohexane) afforded **16**. Yield 74% (1.33 g, 1.89 mmol); R_f = 0.65 (1:9 EtOAc−cyclohexane); $[\alpha]_D$ = +23.3 (*c* 1.0 in $CHCl_3$). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.40, 6.87 (2 d, 4 H, A_2X_2, aromatics), 5.49 (s, 1 H, PhC*H*), 4.78 (d, 1 H, $J_{1,2}$ = 3.8 Hz, H-1), 4.25 (dd, 1 H, $J_{6a,6b}$ = 9.6, $J_{5,6a}$ = 4.6 Hz, H-6a), 3.80 (s, 3 H, OCH_3), 3.76 (m, 1 H, H-5), 3.75−3.59 (m, 6 H, H-3, H6b, OCH_2), 3.47 (t, 1 H, $J_{3,4}$ = $J_{4,5}$ = 9.3 Hz, H-4), 3.42 (s, 3 H, OCH_3), 3.34 (dd, 1 H, $J_{2,3}$ = 9.3, H-2), 1.70−1.49 (m, 4 H, CH_2), 1.22 (bs, 44 H, CH_2), 0.87 (t, 6 H, $^3J_{H,H}$ = 5.8 Hz, CH_3). ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 159.1, 130.0, 127.3, 113.5 (Ph), 101.2 (PhCH), 99.1 (C-1), 81.9 (C-4), 80.4 (C-2), 78.2 (C-3), 73.4, 72.3 (OCH_2), 69.0 (C-6), 62.4 (C-5), 55.2 (OCH_3), 31.9−22.7 (CH_2), 14.1 (CH_3). ESI MS: *m*/*z*: 503.6 [M + Na]$^+$, 519.5 [M + K]$^+$.

Methyl 2,3-Di-O-hexyl-4-O-(p-methoxybenzyl)-α-D-glucopyranoside (17). To a solution of 15 (0.71 g, 1.48 mmol) in a mixture of Et_2O−DCM (2:1, 75 mL), under Ar atmosphere, 1 M $LiAlH_4$ in THF (7.4 mL, 7.40 mmol) and $AlCl_3$ (0.81 g, 6.06 mmol) in Et_2O (25 mL) were added dropwise, and the resulting mixture was refluxed for 4 h. After cooling to rt, EtOAc (250 mL) and H_2O (250 mL) were added. The organic layer was washed with brine (2 × 200 mL), dried ($MgSO_4$), evaporated, and purified by column chromatography (1:2 EtOAc−cyclohexane). Yield 83% (0.60 g, 1.22 mmol); R_f = 0.26 (1:2 EtOAc−cyclohexane); $[\alpha]_D$ = +76.2 (*c* 1.0, DCM). IR: ν_{max}= 1076, 1035 cm^{-1}. ^{1}H NMR (300 MHz, $CDCl_3$): δ =7.26, 6.87 (A_2X_2 system, 4 H, aromatics), 4.81 (d, 1 H, $^2J_{Ha,Hb}$ = 10.8 Hz, PhC*Ha*), 4.75 (d, 1 H, $J_{1,2}$ = 3.5 Hz, H-1), 4.57 (d, 1 H, PhC*Hb*), 3.86 (m, 1 H, OCH_2), 3.79 (s, 3 H, $PhOCH_3$), 3.77−3.64 (m, 3 H, H-6a, H-6b, OCH_2), 3.68 (m, 1 H, H-3), 3.67−3.53 (m, 3 H, OCH_2, H-5), 3.42 (t, 1 H, $J_{3,4}$ = $J_{4,5}$ = 9.5 Hz, H-4), 3.37 (s, 3 H, OCH_3), 3.26 (dd, 1 H, $J_{2,3}$ = 9.5 Hz, H-2), 1.75 (bs, 1 H, OH), 1.64−1.57 (m, 4 H, CH_2), 1.37−1.28 (m, 12 H, CH_2), 0.88 (t, 6 H, $^3J_{H,H}$ = 7.0 Hz, CH_3), 0.87 (t, 6 H, $^3J_{H,H}$ = 6.8 Hz, CH_3). ^{13}C NMR (75.5 MHz, $CDCl_3$): δ =159.3−113.8 (Ph), 98.0 (C-1), 81.6 (C-3), 80.8 (C-2), 77.1 (C-4), 74.5 ($PhCH_2$), 73.7, 71.7 (OCH_2), 70.6 (C-5), 62.0 (C-6), 55.2, 55.0 (OCH_3), 31.8−22.6 (CH_2), 14.0 (CH_3). ESI MS: *m*/*z*: 505.6 [M + Na]$^+$, 521.5 [M + K]$^+$. Anal. Calcd for $C_{27}H_{46}O_7$: C, 67.19; H, 9.61. Found: C, 66.92; H, 9.67.

Methyl 6-Deoxy-2,3-di-O-hexyl-6-iodo-4-O-(p-methoxybenzyl)-α-D-glucopyranoside (19). To a solution of **17** (0.60 g, 1.23 mmol) in toluene (25 mL), TPP (0.49 g, 1.85 mmol) and imidazole (0.25 g, 3.70 mmol) were added. Iodine (0.49 g, 1.73 mmol) was added in portions, and the resulting solution was stirred at 70 °C for 5 h. After cooling at rt, $NaHCO_3$ satd (25 mL) was added and the mixture was stirred for 5 min. Additional iodine was added, and the mixture was stirred for 10 min. Then $Na_2S_2O_3$ aq 10% was added to remove the iodine excess. The organic layer was separated, washed with H_2O (3 × 25 mL), dried ($MgSO_4$), filtered, concentrated, and purified by column chromatography (1:4 EtOAc−cyclohexane). Yield 94% (0.69 g, 1.16 mmol); R_f = 0.52 (1:4 EtOAc−cyclohexane); $[\alpha]_D$ = +84.3 (*c* 1.0, DCM). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.26, 6.88 (A_2X_2 system, 4 H,, aromatics), 4.87 (d, 1 H, $^2J_{Ha,Hb}$ = 10.6 Hz, PhC*Ha*), 4.77 (d, 1 H, $J_{1,2}$ = 3.4 Hz, H-1), 4.62 (d, 1 H, PhC*Hb*), 3.87 (m, 1 H, CH_2), 3.80 (s, 3 H, $PhOCH_3$), 3.75−3.64 (m, 2 H, H-3, OCH_2), 3.63−3.50 (m, 2 H, OCH_2), 3.46 (dd, 1 H, $J_{6a,6b}$ = 10.4 Hz, $J_{5,6a}$ = 2.4 Hz, H-6a), 3.42 (s, 3 H, OCH_3), 3.37 (m, 1 H, H-5), 3.35−3.26 (m, 2 H, H-2, H-6b), 3.24 (t, 1 H, $J_{3,4}$ = $J_{4,5}$ = 9.0 Hz, H-4), 1.66−1.55 (m, 4 H, CH_2), 1.39−1.25 (m, 12 H, CH_2), 0.88 (t, 6 H, $^3J_{H,H}$ = 6.5 Hz, CH_3), 0.87 (t, 6 H, $^3J_{H,H}$ = 6.8 Hz, CH_3). ^{13}C NMR (75.5 MHz, $CDCl_3$): δ =159.4−113.9 (Ph), 98.0 (C-1), 81.2 (C-3), 81.1 (C-2), 80.8 (C-4), 74.9 ($PhCH_2$), 73.7, 71.7 (OCH_2), 69.2 (C-5), 55.4, 55.2 (OCH_3), 31.7−22.6 (CH_2), 14.0 (CH_3), 8.1 (C-6). ESI MS: *m*/*z*: 631.3 [M + K]$^+$, 615.4 [M + Na]$^+$. Anal. Calcd for $C_{27}H_{45}IO_6$: C, 54.73; H, 7.65. Found: C, 54.88; H, 7.71.

Methyl 6-(2-tert-Butoxycarbonylaminoethylthio)-2,3-di-O-hexyl-4-O-p-methoxybenzyl-α-D-glucopyranoside (21). To a suspension of **19** (0.69 g, 1.16 mmol) and Cs_2CO_3 (0.53 g, 1.62 mmol) in dry DMF (10 mL), *tert*-butyl (2-mercaptoethyl)carbamate (0.27 mL, 1.62 mmol) was added and the reaction mixture was stirred at 60 °C, under Ar atmosphere, for 24 h. The mixture was concentrated, EtOAc (25 mL) and water (25 mL) were added, and then the organic layer was separated, washed with H_2O (3 × 25 mL), dried ($MgSO_4$), filtered, and concentrated and the residue was purified by column chromatography (1:6 → 1:4 EtOAc−cyclohexane). Yield 99% (0.74 g, 1.15 mmol); R_f = 0.17 (1:6 EtOAc−cyclohexane); $[\alpha]_D$ = +64.2 (*c* 1.0, DCM). IR: ν_{max} = 1714 cm^{-1}. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.24, 6.87 (2 d, 4 H, $^3J_{H,H}$ = 8.7 Hz, aromatics), 4.98 (bs, 1 H, NH), 4.84 (d, 1 H, $^2J_{Ha,Hb}$ = 10.8 Hz, PhC*Ha*), 4.74 (d, 1 H, $J_{1,2}$ = 3.4 Hz, H-1), 4.54 (d, 1 H, PhC*Hb*), 3.85 (m, 1 H, OCH_2), 3.79 (s, 3 H, $PhOCH_3$), 3.74−3.52 (m, 3 H, OCH_2), 3.71 (m, 1 H, H-5), 3.64 (t, 1 H, $J_{2,3}$ = $J_{3,4}$ = 9.3 Hz, H-3), 3.40 (s, 3 H, OCH_3), 3.31−3.25 (m, 4 H, H-2, H-4, CH_2N), 2.84 (dd, 1 H, $J_{6a,6b}$ = 13.9 Hz, $J_{5,6a}$ = 2.6 Hz, H-6a), 2.71 (t, 2 H, $^3J_{H,H}$ = 6.4 Hz, CH_2S), 2.57 (dd, 1 H, $J_{5,6b}$ = 7.5 Hz, H-6b), 1.65−1.55 (m, 4 H, CH_2), 1.44 (s, 9 H, CMe_3), 1.36−1.24 (m, 12 H, CH_2), 0.88, 0.87 (2 t, 6 H, $^3J_{H,H}$ = 6.6 Hz, CH_3). ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 159.3 (CO), 130.5−113.9 (Ph), 97.8 (C-1), 81.6 (C-3), 80.9 (C-2), 80.1 (C-4), 79.3 (CMe_3), 74.7 (CH_2Ph), 73.7, 71.7 (2 CH_2), 70.7 (C-5), 55.3, 55.0 (2 OCH_3), 39.7 (CH_2N), 33.6 (C-6), 33.5 (CH_2S), 31.7−29.6 (CH_2), 28.4 (CMe_3), 26.9−22.6 (CH_2), 14.0 (CH_3). ESI MS: *m*/*z*: 664 [M + Na]$^+$, 680 [M + K]$^+$. Anal. Calcd for $C_{34}H_{59}NO_8S$: C, 63.62; H, 9.26; N, 2.18; S, 5.00. Found: C, 63.73; H, 9.21; N, 1.98; S, 4.86.

Methyl 6-(2-Aminoethylthio)-2,3-di-O-hexyl-α-D-glucopyranoside Hydrochloride (3). Treatment of **21** (0.35 g, 0.55 mmol) with 1:1 TFA−DCM (2 mL) and freeze-drying from 10:1 H_2O/0.1 N HCl solution afforded **3**. Yield quant (0.20 g, 0.54 mmol); R_f = 0.45 (45:5:3 EtOAc−EtOH−H_2O); $[\alpha]_D$ = +74.4 (*c* 1.0, MeOH). IR: ν_{max}= 3404, 1109 cm^{-1}. ^{1}H NMR (300 MHz, CD_3OD): δ = 4.80 (d, 1 H, $J_{1,2}$ = 3.9 Hz, H-1), 3.74 (t, 1 H, $^3J_{H,H}$ = 6.8 Hz, OCH_2), 3.67−3.51 (m, 4 H, H-5, OCH_2), 3.42 (s, 3 H, OCH_3), 3.40 (t, 1 H, $J_{2,3}$ = $J_{3,4}$ = 9.3 Hz, H-3), 3.29 (t, 1 H, $J_{4,5}$ = 9.3 Hz, H-4), 3.23 (dd, 1 H, H-2), 3.16 (t, 2 H, $^3J_{H,H}$ = 6.8 Hz, CH_2N), 2.99 (dd, 1 H, $J_{6a,6b}$ = 14.4 Hz, $J_{5,6a}$ = 2.3 Hz, H-6a), 2.90 (m, 2 H, CH_2S), 2.72 (dd, 1H, $J_{5,6a}$ = 8.0 Hz, H6b), 1.60−1.52 (m, 4 H, CH_2), 1.42−1.31 (bs, 12 H, CH_2), 0.90, 0.89 (2 t, 6 H, $^3J_{H,H}$ = 6.6 Hz, CH_3). ^{13}C NMR (75.5 MHz, CD_3OD): δ = 99.1 (C-1), 82.6 (C-3), 81.6 (C-2), 74.6 (OCH_2), 74.0 (C-4), 73.4 (C-5), 72.2 (OCH_2), 55.5 (OCH_3), 40.0 (CH_2N), 34.2 (C-6), 33.0−31.1 (CH_2), 31.1 (CH_2S), 26.9, 26.8, 23.7 (CH_2), 14.4 (CH_3). ESI MS: *m*/*z*: 422.5 [M − Cl]$^+$. Anal. Calcd for $C_{21}H_{43}NO_5S$·HCl: C, 55.06; H, 9.68; N, 3.06; S, 7.00. Found: C, 54.87; H, 9.45; N, 2.79; S, 6.78.

Methyl 2,3-Di-O-tetradecyl-α-D-glucopyranoside (18). Methyl 4,6-O-(4-methoxybenzylidene)-2,3-di-O-tetradecyl-α-D-glucopyranoside (0.70 g, 0.98 mmol) was dissolved in a mixture of Et_2O−DCM (2:1, 15 mL) under Ar atmosphere. $AlCl_3$ (0.81 g, 6.06 mmol) in Et_2O (15 mL) was added dropwise, and the resulting mixture was refluxed for 4 h. After cooling to rt, EtOAc (150 mL) and H_2O (150 mL) were added and the layers separated. The organic layer was washed with brine (3 × 100 mL), dried (Mg_2SO_4), and evaporated. Column chromatography of the residue (1:1 EtOAc−cyclohexane) afforded **18**. Yield 87% (0.50 mg, 0.85 mmol); R_f = 0.47 (1:1 EtOAc−cyclohexane); $[\alpha]_D$ = +24.7 (*c* 1.0, DCM). IR: ν_{max} 3362, 2953, 1468 cm^{-1}. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 4.78 (d, 1 H, $J_{1,2}$ = 3.5 Hz, H-1), 3.94−3.43 (m, 9 H, H-3, H,4 H-5, H-6a, H-6b, 2 OCH_2), 3.41 (s, 3 H, OCH_3), 3.26 (dd, 1 H, $J_{2,3}$ = 9.2 Hz, H-2), 1.69−1.48 (m, 4 H, CH_2), 1.25 (bs, 44 H, CH_2), 0.87 (t, 6 H, $^3J_{H,H}$ = 6.9 Hz, CH_3). ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 98.1 (C-1), 81.0, 80.6 (C-2, C-3), 73.6 (C-4), 71.3, 70.7 (OCH_2), 70.5 (C-5), 62.5 (C-6), 55.2 (OCH_3), 31.9−22.6 (CH_2), 14.1 (CH_3). ESI MS: *m*/*z*: 625.6 [M + K]$^+$, 609.8

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$[M + Na]^+$. Anal. Calcd for $C_{35}H_{70}O_6$: C, 71.62; H, 12.02. Found: C, 71.38; H, 11.76.

*Methyl 2,3-Di-O-tetradecyl-6-deoxy-6-iodo-α-D-glucopyranoside (**20**).* To a solution of **18** (0.49 g, 0.84 mmol) in toluene (17 mL), triphenylphosphine (0.33 g, 1.26 mmol) and imidazole (0.17 g, 2.52 mmol) were added. Iodine (0.33 g, 1.17 mmol) was added in portions, and the resulting solution was stirred at 70 °C for 3 h. After cooling at rt, $NaHCO_3$ satd (20 mL) was added and the mixture was stirred for 5 min. Additional iodine was added up to turn the organic phase brown, and the mixture was stirred for 10 min. Then aq 10% $Na_2S_2O_3$ was added to remove the iodine excess. The organic layer was separated, washed with H_2O (3 × 20 mL), dried ($MgSO_4$), filtered, concentrated, and purified by column chromatography (1:9 EtOAc−cyclohexane). Yield 91% (0.03 g, 0.76 mmol; R_f = 0.30 (1:9 EtOAc−cyclohexane); $[\alpha]_D$ = +44.1 (*c* 1.0, DCM). IR: ν_{max}= 1041, 722 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$): δ = 4.81 (d, 1 H, $J_{1,2}$ = 3.6 Hz, H-1), 3.91 (m, 1 H, OCH_2), 3.66−3.49 (m, 5 H, H-6a, OCH_2, H-3), 3.47 (s, 3 H, OCH_3), 3.45 (ddd, 1 H, $J_{4,5}$ = 9.2 Hz, $J_{5,6a}$ = 6.8 Hz, $J_{5,6b}$ = 2.2 Hz, H-5), 3.31 (m, 1 H, H-4) 3.29 (dd, 1 H, $J_{2,3}$ = 10.0 Hz, H-2), 3.26 (dd, 1 H, $J_{6a,6b}$ = 11.2 Hz, H-6b), 2.41 (d, 1 H, $J_{4,OH}$ = 2.3 Hz, OH-4), 1.61−1.54 (m, 4 H, CH_2), 1.25 (bs, 44 H, CH_2), 0.88 (t, 6 H, $^3J_{H,H}$ = 6.9 Hz, CH_3). ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 98.2 (C-1), 80.7, 80.5 (C-2, C-3), 73.8 (C-4), 73.6, 71.2 (OCH_2), 69.7 (C-5), 55.5 (OCH_3), 31.9−22.7 (CH_2), 14.1 (CH_3), 7.2 (C-6). ESI MS: *m*/*z*: 735.6 $[M + K]^+$, 719.7 $[M + Na]^+$. Anal. Calcd for $C_{35}H_{69}IO_5$: C, 60.33; H, 9.98. Found: C, 59.89; H, 9.72.

*Methyl 6-(2-tert-Butoxycarbonylaminoethylthio)-2,3-di-O-tetradecyl-α-D-glucopyranoside (**22**).* To a suspension of **20** (0.53 g, 0.77 mmol) and Cs_2CO_3 (0.35 g, 1.07 mmol) in DMF (7 mL), *tert*-butyl (2-mercaptoethyl)carbamate (0.18 mL, 1.07 mmol) was added and the reaction mixture was stirred at 60 °C, under Ar atmosphere, for 24 h. The mixture was concentrated then EtOAc (20 mL) and H_2O (20 mL) were added, and the organic layer was separated, washed with H_2O (3 × 20 mL), dried ($MgSO_4$), filtered, and concentrated. The residue was purified by column chromatography (1:3 EtOAc−cyclohexane), affording **22**. Yield 95% (0.54 g, 0.73 mmol); R_f = 0.40 (1:3 EtOAc−cyclohexane); $[\alpha]_D$ = +50.4 (*c* 1.0, DCM). IR: ν_{max}= 3631, 1698 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$): δ = 4.99 (bs, 1 H, NH), 4.77 (d, 1 H, $J_{1,2}$ = 3.6 Hz, H-1), 3.90 (m, 1 H, OCH_2), 3.72 (ddd, 1 H, $J_{4,5}$ = 9.3 Hz, $J_{5,6a}$ = 7.5 Hz, $J_{5,6b}$ = 2.6 Hz, H-5), 3.64−3.46 (m, 3 H, OCH_2), 3.48 (t, 1 H, $J_{2,3}$ = $J_{3,4}$ = 9.3 Hz, H-3), 3.43 (s, 3 H, OCH_3), 3.36 (t, 1 H, H-4), 3.31 (m, 2 H, CH_2N), 3.27 (dd, 1 H, H-2), 2.98 (dd, 1 H, $J_{6a,6b}$ = 14.1 Hz, H-6a), 2.71 (m, 3 H, H6b, CH_2S), 2.63 (bs, 1 H, OH-4), 1.65−1.50 (m, 4 H, CH_2), 1.43 (s, 9 H, CMe_3), 1.24 (bs, 44 H, CH_2), 0.87 (t, 6 H, $^3J_{H,H}$ = 7.0 Hz, CH_3). ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 156 (CO), 97.9 (C-1), 80.9 (C-3), 80.6 (C-2), 79.4 (CMe_3), 73.5 (OCH_2), 72.4 (C-4), 71.2 (OCH_2), 71.0 (C-5), 55.2 (OCH_3), 39.6 (CH_2N), 33.5 (C-6), 33.4 (CH_2S), 31.8−29.3 (CH_2), 28.4 (CMe_3), 26.1−22.6 (CH_2), 14.0 (CH_3). ESI MS: *m*/*z*: 784.7 $[M + K]^+$, 768.8 $[M + Na]^+$. Anal. Calcd for $C_{42}H_{83}NO_7S$: C, 67.60; H, 11.21; N, 1.88; S, 4.30. Found: C, 67.45; H, 10.90; N, 1.62; S, 4.24.

*Methyl 6-(2-Aminoethylthio)-2,3-di-O-tetradecyl-α-D-glucopyranoside Hydrochloride (**4**).* Treatment of **22** (0.25 g, 0.33 mmol) with 1:1 TFA−DCM (2 mL) and freeze-drying from 10:1 H_2O/0.1 N HCl solution afforded **4**. Yield quant (0.22 g,, 0.33 mmol). Column chromatography of the residue (4:1 EtOAc−cyclohexane → EtOAc → 45:5:3 EtOAc−EtOH−H_2O). R_f = 0.25 (45:5:3 EtOAc−EtOH−H_2O); $[\alpha]_D$ = +41.3 (*c* 0.9, 9:1 DCM−MeOH). IR: ν_{max}= 3406, 722 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$): δ = 8.11 (bs, 2 H, NH_2HCl), 4.78 (d, 1 H, $J_{1,2}$ = 3.2 Hz, H-1), 3.87 (m, 1 H, OCH_2), 3.74 (ddd, 1 H, $J_{4,5}$ = 9.4 Hz, $J_{5,6b}$ = 6.7 Hz, $J_{5,6a}$ = 2.7 Hz, H-5), 3.66−3.54 (m, 3 H, OCH_2), 3.49 (t, 1 H, $J_{2,3}$ = $J_{3,4}$ = 8.9 Hz, H-3), 3.42 (s, 3 H, OCH_3), 3.38 (t, 1 H, H-4), 3.27 (dd, 1 H, H-2), 3.18 (t, 2 H, $^3J_{H,H}$ = 6.2 Hz, CH_2N), 2.97 (dd, 1 H, $J_{6a,6b}$ = 14.2 Hz, H-6a), 2.90 (m, 2 H, CH_2S), 2.74 (dd, 1H, H6b), 1.60−1.54 (m, 4 H, CH_2), 1.25 (bs, 44 H, CH_2), 0.87 (t, 6 H, $^3J_{H,H}$ = 6.9.0 Hz, CH_3). ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 97.9 (C-1), 80.9 (C-3), 80.5 (C-2), 73.7 (OCH_2), 72.1 (C-4), 71.3 (OCH_2), 70.7 (C-5), 55.2 (OCH_3), 38.7 (CH_2N), 33.2 (C-6), 31.9 (CH_2), 30.3 (CH_2S), 30.3−22.7 (CH_2), 14.1 (CH_3). ESI MS: *m*/*z*: 646.7 $[M - HCl]^+$. Anal. Calcd for $C_{37}H_{75}NO_5S{\cdot}HCl{\cdot}2H_2O$: C, 61.85; H, 11.22; N, 1.95; S, 4.46. Found: C, 61.79; H, 11.03; N, 2.01; S, 4.43.

*Methyl 6-(2-tert-Butoxycarbonylaminoethylthio)-2,3,4-tri-O-hexanoyl-α-D-glucopyranoside (**24**).* To a solution of **23** (0.49 g, 0.83 mmol) in dry DMF (7.5 mL), Cs_2CO_3 (0.38 g, 0.16 mmol) and *tert*-butyl *N*-(2-mercaptoethyl)carbamate (196 μL, 1.16 mmol) were added, under Ar atmosphere, and the reaction mixture was stirred at 60 °C for 48 h. The reaction mixture was concentrated, and the crude product was dissolved in DCM (10 mL) and washed with H_2O (2 × 15 mL). The organic phase was dried ($MgSO_4$), filtered, and concentrated, and the residue was purified by flash column chromatography (1:4 EtOAc−cyclohexane). Yield 85% (0.45 mg, 0.70 mmol); R_f = 0.38 (1:3 EtOAc−cyclohexane); $[\alpha]_D$ = +62.1 (*c* 1.0, DCM). IR: ν_{max} = 2958, 1747, 1701 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$): δ = 5.46 (t, 1 H, $J_{2,3}$ = $J_{3,4}$ = 10 Hz, H-3), 4.95 (t, 1 H, $J_{4,5}$= 10.0 Hz, H-4), 3.41 (s, 3 H, OCH_3), 4.90 (d, 1 H, $J_{1,2}$ = 3.7 Hz, H-1), 4.83 (dd, 1 H, H-2), 3.90 (dt, 1 H, $J_{5,6b}$ = 8.0 Hz, H-5), 3.27 (q, 2 H, $J_{H,H}$ = 6.0 Hz, CH_2N), 2.80−2.50 (m, 4 H, CH_2S, H-6a, H-6b), 2.52 (m, 2 H, H-6a, H-6b), 2.31 (m, 6 H, H-2_{Hex}), 1.54 (m, 6 H, H-3_{Hex}), 1.41 (s, 9 H, CMe_3), 1.25 (m, 12 H, H-4_{Hex}, H-5_{Hex}), 0.86 (t, 9 H, H-6_{Hex}). ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 172.9−172.0 (CO ester), 155.7 (CO carbamate) 96.7 (C-1), 71.5 (C-4), 70.9 (C-2), 69.3 (C-3), 69.9 (C-5), 55.1 (OMe), 39.58 (CH_2N), 33.7 (CH_2S), 33.0 (C-6), 34.0 (C-2_{Hex}), 24.5 (C-3_{Hex}), 28.2 (CMe_3), 22.1 (C-5_{Hex}), 13.6 (C-6_{Hex}). ESI MS: *m*/*z* = 670.4 $[M + Na]^+$. Anal. Calcd for $C_{32}H_{57}NO_{10}S$: C, 59.32; H, 8.87; N, 2.16; S, 4.95. Found: C, 59.45; H, 8.93; N, 2.31; S, 4.71.

*Methyl 6-(2-Aminoethylthio)-2,3,4-tri-O-hexanoyl-α-D-glucopyranoside Hydrochloride (**25**).* Treatment of **24** (0.29 g, 0.44 mmol) with 1:1 TFA−DCM (6 mL) and freeze-drying from 10:1 H_2O/0.1 N HCl solution afforded **25**. Yield quant (0.27 mg, 0.44 mmol); $[\alpha]_D$ = +72.0 (*c* 0.7, EtOAc). IR: ν_{max} = 2962, 1749 cm^{-1}. ^1H NMR (300 MHz, CD_3OD): δ = 8.05 (bs, 3 H, NH_3), 5.28 (t, 1 H $J_{2,3}$ = 9.7 Hz, $J_{3,4}$ = 9.7 Hz, H-3), 4.94 (t, 1 H, $J_{4,5}$ = 9.7 Hz, H-4), 4.88 (m, 1 H, $J_{1,2}$ = 3.8 Hz, H-1), 4.82 (dd, 1 H, H-2), 3.8 (m, 1 H, H-5), 3.34 (s, 1 H, OCH_3), 2.93 (bs, 2 H, CH_2N), 2.78 (m, 2 H, CH_2S), 2.73 (dd, 1 H, $J_{6a,6b}$ = 14.0 Hz, $J_{5,6a}$ = 2.7, H-6a), 2.62 (dd, 1 H, $J_{5,6b}$ = 7.7 Hz, H-6b), 2.26 (m, 6 H, H-2_{Hex}), 1.44 (m, 6 H, H-3_{Hex}), 1.21 (m, 12 H, H-4_{Hex}, H-5_{Hex}), 0.82 (t, 9 H, H-6_{Hex}). ^{13}C NMR (75.5 MHz, CD_3OD): δ = 172−171 (CO), 95.98 (C-1), 71.2 (C-4), 70.6 (C-2), 69.4 (C-3), 69.1 (C-5), 55.1 (OMe), 39.1 (CH_2N), 32.5 (C-6), 30.5 (CH_2S), 33.1 (C-2_{Hex}), 31.1 (C-4_{Hex}), 24 (C-3_{Hex}), 22.1(C-2_{Hex}), 14.4 (C-6_{Hex}). ESIMS: *m*/*z* = 548.3 $[M + H]^+$. Anal. Calcd for $C_{27}H_{50}ClNO_8S{\cdot}HCl$: C, 55.51; H, 8.63; N, 2.40; S, 5.49. Found: C, 55.37; H, 8.62; N, 2.26; S, 5.27.

*Methyl 6-(2-(N′-(2-(N,N-Di-(2-(N-tert-butoxycarbonylamino)-ethyl)amino)ethyl)thioureido)-ethylthio)-2,3,4-tri-O-hexanoyl-α-D-glucopyranoside (**27**).* To a solution of **25** (0.12 g, 0.20 mmol) and Et_3N (56 μL, 0.4 mmol) in DCM (6 mL), 2-[*N*,*N*-bis(2-(*N*-*tert*-butoxyaminocarbonyl)ethylamino]ethyl isothiocyanate (0.09 g, 0.24 mmol) was added and the reaction mixture was stirred, under Ar atmosphere, at rt for 48 h. The reaction mixture was washed with aqueous diluted HCl (2 × 20 mL), dried ($MgSO_4$), filtered, and concentrated. The residue was purified by column chromatography (1:1→ 3:1 EtOAc−cyclohexane). Yield 50% (0.08 g, 0.10 mmol); R_f = 0.2 (1:1 EtOAc−cyclohexane); $[\alpha]_D$ = +71.6 (*c* 1.0, DCM). IR: ν_{max} = 2959, 1748, 1685 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$): δ = 7.46, 6.96 (2 bs, 2 H, NHCS), 5.46 (t, 1 H, $J_{2,3}$ = $J_{3,4}$ = 9.8 Hz, H-3), 4.96 (t, 1 H, $J_{4,5}$ = 9.8 Hz, H-4), 4.90 (d, 1 H, $J_{1,2}$ = 3.5 Hz, H-1), 4.88 (bs, 2 H, NHBoc), 4.84 (dd, 1 H, H-2), 3.93 (bd, 1 H, H-5), 3.77 (q, 2 H, $NHCH_2CH_2N$), 3.52 (bs, 2 H, SCH_2CH_2N), 3.42 (s, 3 H, OCH_3), 3.11 (q, 4 H, NCH_2CH_2NHBoc), 2.82 (t, 2 H, $J_{H,H}$ = 7.0 Hz, $NHCH_2CH_2N$), 2.77−2.56 (m, 4 H, H-6a, H-6b, SCH_2), 2.50 (bs, 4 H, NCH_2CH_2NHBoc), 2.37−2.1 (m, 6 H, H-2_{Hex}), 1.57 (m, 6 H, $J_{H,H}$ = 7 Hz, H-3_{Hex}), 1.43 (s, 9 H, CMe_3), 1.27 (m, 12 H, H-4_{Hex}, H-5_{Hex}), 0.87 (t, 9 H, H-6_{Hex}). ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 182.1 (CS), 172.0−171.0 (CO ester), 155.2 (CO carbamate), 96.6 (C-1), 70.9 (C-4), 70.1 (C-2), 69.9 (C-5), 69.6 (C-3), 55.8 (NCH_2CH_2NHBoc), 55.5 (OMe), 54.0 (SCH_2), 44.1 ($NHCH_2CH_2N$), 42.4 (SCH_2CH_2N), 39.4 (NCH_2CH_2NHBoc), 33.1 (C-6), 32.6 ($NHCH_2CH_2N$), 34.1 (C-2_{Hex}), 31.2 (C-5 $_{Hex}$), 28.4 (CMe_3) 24.5 (C-3_{Hex}), 22.1 (C-5_{Hex}), 13.9

(C-6_{Hex}). ESI MS: m/z = 958.6 $[M + Na]^+$ Anal. Calcd for $C_{44}H_{81}N_5O_{12}S_2$: C, 56.44; H, 8.72; N, 7.48; S, 6.85. Found: C, 56.61; H, 8.89; N, 7.21; S, 6.60.

*Methyl 6-(2-(N′-(2-(N,N-Bis(2-aminoethyl)amino)ethyl)-thioureido)ethylthio)-2,3,4-tri-O-hexanoyl-α-D-glucopyranoside Trihydrochloride (**5**).* Treatment of **27** (0.15 g, 0.16 mmol) with 1:1 TFA–DCM (2 mL) and freeze-drying from 10:1 H_2O/0.1 N HCl solution afforded **5**. Yield quant (0.13 g, 0.16 mmol). $[\alpha]_D$ = +46.8 (*c* 0.85, MeOH). IR: ν_{max} = 2958, 1747, 1675 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$): δ = 7.98 (bs, 4 H, NHCS, NH_2HCl), 7.60 (bs, 1 H, NHCS), 5.47 (t, $J_{3,4} = J_{2,3}$= 9.5 Hz, 1 H, H-3), 4.99 (t, 1 H, $J_{4,5}$ = 9.5 Hz, H-4), 4.91 (dd, 1 H, $J_{1,2}$ = 4 Hz, H-1), 4.85 (dd, 1 H, H-2), 3.96 (td, 1 H, $J_{5,6b}$ = 7.3 Hz, $J_{5,6a}$ = 2.4 Hz, H-5), 3.73 (bs, 2 H, SCH_2CH_2NHCS), 3.68 (bs, 2 H, $NHCSCH_2CH_2N$), 3.41 (s, 3 H, OCH_3), 3.11 (bs, 4 H, CH_2NH_3Cl), 2.84 (bs, 6 H, $CH_2CH_2NH_3Cl$, SCH_2CH_2NHCS), 2.75 (m, 3 H, H-6a, $NHCSCH_2CH_2N$), 2.66 (dd, 1 H, $J_{6a,6b}$ = 13.8 Hz, H-6b), 2.32–2.19 (m, 6 H, H-2_{Hex}), 1.55 (m, 6 H, H-3_{Hex}), 1.27 (m, 12 H, H-4_{Hex}, H-5_{Hex}), 0.87 (m, 9 H, H-6_{Hex}). ^{13}C NMR (75 MHz, $CDCl_3$): 173.1, 173.0, 172.6 (CO), 96.6 (C-1), 71.2 (C-4), 70.8 (C-2), 69.6 (C-3), 69.1 (C-5), 55.4 (OCH_3), 53.4 ($NHCSCH_2CH_2N$), 52.0 ($CH_2CH_2NH_2HCl$), 43.1 (SCH_2CH_2NHCS), 41.7 ($NHCSCH_2CH_2N$), 37.6 (CH_2NH_3Cl), 34.1, 34.0 (C-2_{Hex}), 33.0 (C-6), 32.7 (SCH_2CH_2NHCS), 31.2, 31.1 (C-4_{Hex}), 24.5, 24.4 (C-3_{Hex}), 22.2 (C-5_{Hex}), 13.8 (C-6_{Hex}). ESI MS: m/z = 736.4 $[M]^+$. Anal. Calcd for $C_{34}H_{65}N_5O_8S_2{\cdot}3HCl$: C, 48.30; H, 8.11; N, 8.28; S, 7.58. Found: C, 48.24; H, 8.39; N, 8.15; S, 7.41.

*Methyl 6-(4-(2,2-Bis-tert-butoxycarbonylamino)-ethylaminomethyl)-1H-1,2,3-triazol-1-yl-6-deoxy)-2,3,4-tri-O-hexanoyl-α-D-glucopyranoside (**29**).* To a solution of **28** (0.20 g, 0.39 mmol) and 3-bis[2-*tert*-butoxycarbonylamino)ethyl]propargylamine (0.29 g, 0.85 mmol) in H_2O–^{t}BuOH 9:1 (15 mL), the Cu-supported catalyst Si-BPA·Cu^+ (0.02g) was added and the reaction mixture was refluxed for 36 h at 85 °C. The catalyst was filtered, and the solvent was removed. The residue was purified by column chromatography (1:1 → 2:1 EtOAc–cyclohexane). Yield 78% (0.25 g, 0.30 mmol). R_f = 0.61 (9:1 DCM–MeOH); $[\alpha]_D$ = +50.5 (*c* 1.0, DCM). IR: ν_{max} = 2957, 2359, 1748, 1703, 734 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$): δ = 7.59 (s, 1 H, =CH), 5.49 (t, 1 H, $J_{2,3} = J_{3,4}$ = 10.3 Hz, H-3), 4.89 (bs, 2 H, NHBoc), 4.87 (d, 1 H, $J_{1,2}$ = 3.5 Hz, H-1), 4.85 (t, 1 H, $J_{4,5}$ = 10.3 Hz, H-4), 4.81 (dd, 1 H, H-2), 4.53 (dd, 1 H, $J_{6a,6b}$ = 14.0 Hz, $J_{5,6a}$ = 2.6 Hz, H-6a), 4.29 (dd, 1 H, $J_{5,6b}$ = 9.0 Hz, H-6b), 4.17 (ddd, 1 H, H-5), 3.80 (m, 2 H, CH_2-triazole), 3.18 (bs, 4 H, CH_2NHBoc), 3.07 (s, 3 H, OCH_3), 2.55 (t, 4 H, CH_2CH_2NHBoc), 2.27 (m, 6 H, H-2_{Hex}), 1.58 (s, 9 H, CMe_3), 1.44 (m, 6 H, H-3_{Hex}), 1.30 (m, 12 H, H-4_{Hex},H-5_{Hex}), 0.89 (m, 9 H, H-6_{Hex}). ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 173.9, 173.5, 173,1 (CO ester), 156.2 (CO carbamate), 144.1 (C-4 triazole), 124.3 (C-5 triazole), 91.4 (C-1), 78.1 (CMe_3), 70.6 (C-2), 69.8 (C-4), 69.3 (C-3), 68.0 (C-5), 53.1 (CH_2CH_2NHBoc), 50.6 (C-6), 48.3 (CH_2 triazole), 38.4 (CH_2NHBoc), 34.1 (C-2_{Hex}), 31.7 (C-4_{Hex}), 28.5 (CMe_3), 24.6 (C-3_{Hex}), 22.6 (C-5_{Hex}), 13.8 (C-6_{Hex}). ESI MS: m/z = 877.5 $[M + Na]^+$. Anal. Calcd for $C_{42}H_{74}N_6O_{12}$: C, 59.00; H, 8.72; N, 9.83. Found: C, 59.09; H, 8.77; N, 9.64.

*Methyl 6-Deoxy-6-(4-(2,2-diaminoethylaminomethyl)-1H-1,2,3-triazol-1-yl)-2,3,4-tri-O-hexanoyl-α-D-glucopyranoside Dihydrochloride (**30**).* Treatment of **29** (0.42 g, 0.49 mmol) with 1:1 TFA–DCM (5 mL) and freeze-drying from 10:1 H_2O/0.1 N HCl solution afforded **30**. Yield quant (0.35 g, 0.49 mmol); $[\alpha]_D$ = +38.1 (*c* 1.0, MeOH). IR: ν_{max} = 2957, 1748, 1675 cm^{-1}. ^1H NMR (300 MHz, CD_3OD): δ = 8.07 (s, 1 H, =CH), 5.42 (t, 1 H, $J_{2,3} = J_{3,4}$ = 9.5 Hz, H-3), 4.93 (d, 1 H, $J_{1,2}$ = 3.5 Hz, H-1), 4.82 (dd, 1 H, H-2), 4.76 (t, 1 H, $J_{4,5}$ = 9.5 Hz, H-4), 4.64 (m, 2 H, H-6a, H-6b), 4.27 (ddd, 1 H, $J_{5,6a}$ = 3.7 Hz, $J_{5,6b}$ = 6.0 Hz, H-5), 3.92 (s, 2 H, CH_2 triazole), 3.26 (s, 3 H, OMe), 3.16 (t, 4 H, $J_{H,H}$ = 6.4 Hz, $CH_2CH_2NH_2$), 2.82 (t, 4 H, $CH_2CH_2NH_2$), 2.48–2.15 (m, 6 H, H-2_{Hex}), 1.58 (m, 6 H, H-3_{Hex}), 1.33 (m, 12 H, H-4_{Hex}, H-5_{Hex}), 0.92 (m, 9 H, H-6_{Hex}). ^{13}C NMR (75.5 MHz, CD_3OD): δ = 174.2–173.9 (CO), 143.8 (C-4 triazole), 126.9 (C-5 triazole), 98.6 (C-1), 71.8 (C-2), 70.7 (C-3), 70.3 (C-4), 68.9 (C-5), 56.0 (OCH_3), 52.0 ($CH_2CH_2NH_2$), 51.6 (C-6), 47.4 (CH_2 triazole), 38.2 ($CH_2CH_2NH_2$), 34.9, 34.8, 34.7 (C-2_{Hex}), 32.4, 32.2 (C-4_{Hex}), 25.6, 25.5 (C-3_{Hex}), 23.4 (C-5_{Hex}), 14.2 (C-6_{Hex}). ESI MS: m/z = 831.3 $[M + TFA + Cl + Cu]^+$; 717.3 $[M + Cu + Cl]^+$. Anal. Calcd for $C_{32}H_{59}N_6O_8{\cdot}2HCl$: C, 52.81; H, 8.31; N, 11.55. Found: C, 52.69; H, 8.1; N, 11.72.

*Dendritic Boc-Protected Diminoethyl-bis(thiourea) Glucopyranoside Derivative **31**.* To a solution of **30** (0.20 g, 0.27 mmol) and Et_3N (115 μL, 0.82 mmol) in DCM (12 mL), *tert*-butyl *N*-(2-isothiocyanoethyl)carbamate (0.17 mg, 0.82 mmol) was added and the mixture was stirred overnight at rt. The reaction mixture was washed with aqueous diluted HCl (3 × 10 mL), and the organic phase was dried ($MgSO_4$), filtered, and concentrated. The residue was purified by column chromatography (3:1 EtOAc–cyclohexane → 20:1 DCM–MeOH). Yield 52% (0.15 g, 0.14 mmol); R_f = 0.44 (9:1 DCM–MeOH); $[\alpha]_D$ = +31.7 (*c* 1.0, DCM). IR: ν_{max} = 2959, 1750, 1698, 736 cm^{-1}. UV (DCM): 248 nm (ε_{mM} 47.8). ^1H NMR (300 MHz, $CDCl_3$): δ = 7.62 (s, 1 H, =CH), 7.18, 6.94 (bs, 4 H, NHCS), 5.49 (t, 1 H, $J_{2,3} = J_{3,4}$ = 9.5 Hz, H-3), 5.38 (bs, 2 H, NHBoc), 4.82 (d, 1 H, $J_{1,2}$ = 3.5 Hz, H-1), 4.80 (t, 1 H, $J_{4,5}$ = 9.5 Hz, H-4), 4.80 (dd, 1 H, H-2), 4.53 (dd, 1 H, $J_{6a,6b}$ = 14.5 Hz, $J_{5,6a}$ = 2.6 Hz, H-6a), 4.37 (dd, 1 H, $J_{5,6b}$ = 8.0 Hz, H-6b), 4.17 (ddd, 1 H, H-5), 3.79 (s, 2 H, CH_2 triazole), 3.62 (bs, 4 H, CH_2CH_2NHBoc), 3.54 (bs, 4 H, NCH_2CH_2NHCS), 3.31 (m, 4 H, CH_2NHBoc), 3.12 (s, 1 H, OCH_3), 2.69 (bs, 4 H, NCH_2CH_2NHCS), 2.42–2.13 (m, 6 H, H-2_{Hex}), 1.55 (m, 6 H, H-3_{Hex}), 1.42 (s, 18 H, CMe_3), 1.26 (m, 12 H, H-4_{Hex}, H-5_{Hex}), 0.87 (t, 9 H, H-6_{Hex}). ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 182.7 (CS), 173.0–172.5 (CO ester), 155.5 (CO carbamate), 144.5 (C-4 triazole), 124.4 (C-5 triazole), 96.7 (C-1), 79.8 (CMe_3), 70.2 (C-2), 69.6 (C-4), 69.2 (C-3), 67.8 (C-5), 55.4 (OMe), 52.4 (NCH_2CH_2NHCS), 50.7 (C-6), 48.0 (CH_2 triazole), 44.6 (CH_2CH_2NHBoc), 42.1 (NCH_2CH_2NHCS), 40.1 (CH_2NHBoc), 34.3 (C-2_{Hex}), 30.8 (C-4_{Hex}), 28.1 (CMe_3), 24.2 (C-3_{Hex}), 22.6 (C-5_{Hex}), 14.1 (C-6_{Hex}). ESI MS: m/z = 1081.5 $[M + Na]^+$. Anal. Calcd for $C_{48}H_{86}N_{10}O_{12}S_2$: C, 54.42; H, 8.18; N, 13.22; S, 6.05. Found: C, 54.37; H, 7.98; N, 13.28; S, 5.85

*Dendritic Diaminoethyl-bis(thiourea) Glucopyranoside Dihydrochloride Derivative (**6**).* Treatment of **31** (0.12 g, 0.12 mmol) with 1:1 TFA–DCM (2 mL) and freeze-drying from 10:1 H_2O/0.1 N HCl solution afforded **6**. Yield 91% (0.10 g, 0.11 mmol); $[\alpha]_D$ = +47.7 (*c* 1.0, MeOH). IR: ν_{max} = 2955, 1748, 1676 cm^{-1}. UV (MeOH): 244 nm (ε_{mM} 29.1). ^1H NMR (300 MHz, CD_3OD): δ = 8.43 (s, 1 H, =CH), 5.43 (t, 1 H, $J_{2,3} = J_{3,4}$ = 9.7 Hz, H-3), 4.97 (d, 1 H, $J_{1,2}$ = 3.5 Hz, H-1), 4.90 (dd, 1 H, H-2), 4.82 (t, 1 H, $J_{4,5}$ = 9.7 Hz, H-4), 4.77 (m, 2 H, CH_2 triazole), 4.70 (m, 2 H, H-6a, H-6b), 4.27 (ddd, 1 H, $J_{5,6a}$ = 3.7 Hz, $J_{5,6b}$ = 5.6 Hz, H-5), 4.06 (bs, 4 H, $CH_2CH_2NH_2$), 3.87 (t, 4 H, $^3J_{H,H}$ = 5.8 Hz, NCH_2CH_2NHCS), 3.52 (t, 4 H, $^3J_{H,H}$ = 5.8 Hz, CH_2NH_2), 3.25 (s, 3 H, OCH_3), 3.21 (t, 4 H, $J_{H,H}$ = 6.0 Hz, NCH_2CH_2NHCS), 2.50–2.15 (m, 6 H, H-2_{Hex}), 1.56 (m, 6 H, H-3_{Hex}), 1.31 (m, 12 H, H-4_{Hex}, H-5_{Hex}), 0.91 (m, 9 H, H-6_{Hex}). ^{13}C NMR (75.5 MHz, CD_3OD): δ = 184.1 (CS), 174.3–173.9 (CO), 137.2 (C-4 triazole), 130.1 (C-5 triazole), 98.2 (C-1), 71.8 (C-2), 71.2 (C-3), 70.5 (C-4), 68.7 (C-5), 56.2 (OMe), 54.4 (CH_2NH_2), 51.4 (C-6), 48.4 (CH_2 triazole), 42.2 ($CH_2CH_2NH_2$), 40.4 (NCH_2CH_2NHCS), 40.0 (CH_2NH_2), 34.4 (C-2_{Hex}), 32.0 (C-4_{Hex}), 25.2 (C-3_{Hex}), 22.8 (C-5_{Hex}), 14.0 (C-6_{Hex}). ESI MS: m/z = 859.5 $[M + Na]^+$; 921.4 $[M + Cu]^+$. Anal. Calcd for $C_{38}H_{72}Cl_2N_{10}O_8S_2$: C, 48.97; H, 7.79; N, 15.03; S, 6.88. Found: C, 48.71; H, 77.40; N, 15.23; S, 6.65.

*2,3,4,2′,3′,4′-Hexa-O-hexyl-6,6′-di-O-trityl-α,α′-trehalose (**33**).* To a solution of **32** (1.00 g, 1.21 mmol) in dry DMF (11 mL), NaH (871 mg, 21.78 mmol) was added and the mixture was stirred at 0 °C for 10 min. 1-Bromohexane (3.06 mL, 21.78 mmol) was added dropwise under Ar atmosphere, and the mixture was stirred overnight at rt. The reaction was quenched with MeOH (5 mL) and stirred for 10 min. The solvents were removed, and the resulting residue was suspended in DCM (50 mL) and washed with H_2O (3 × 15 mL), and the organic layer was separated, dried ($MgSO_4$), filtered, concentrated, and purified by column chromatography (1:8 → 1:6 EtOAc–cyclohexane). Yield 92% (1.50 g, 1.11 mmol); R_f = 0.74 (1:5 EtOAc–cyclohexane); $[\alpha]_D$ = +70.3 (*c* 1.0, DCM). IR: ν_{max} = 2923, 2855 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$): δ = 7.53–7.23 (m, 30 H, Ph), 5.34 (d, 2 H, $J_{1,2}$ = 3.7 Hz, H-1), 4.03 (bd, 2 H, $J_{4,5}$ = 9.3 Hz, H-5), 3.80 (m, 2 H, OCH_2),

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3.78−3.41 (m, 8 H, OCH_2), 3.57 (t, 2 H, $J_{2,3} = J_{3,4} = 9.3$ Hz, H-3), 3.51−3.41 (m, 4 H, H-4, H-6a), 3.39 (dd, 2 H, H-2), 3.24 (m, 2 H, OCH_2), 3.13 (dd, 2 H, $J_{6a,6b} = 10.0$ Hz, $J_{5,6b} = 3.3$ Hz, H-6b), 1.65−1.56 (m, 12 H, CH_2), 1.38−1.04.89 (m, 36 H, CH_2), 0.93−0.82 (m, 18 H, CH_3). ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 144.0, 128.8, 127.6, 126.8 (Ph), 93.6 (C-1), 86.1 (Ph_3C), 81.5 (C-3), 80.6 (C-2), 78.1 (C-4), 73.7, 73.0, 71.3 (OCH_2), 70.0 (C-5), 62.0 (C-6), 31.8−22.5 (CH_2), 14.1 (CH_3). ESI MS: m/z = 1353.8 $[M + Na]^+$. Anal. Calcd for $C_{86}H_{122}O_{11}$: C, 77.55; H, 9.23. Found: C, 77.67; H, 9.31.

*2,3,4,2′,3′,4-Hexa-O-tetradecyl-6,6′-di-O-trityl-α,α′-trehalose (**34**).* To a solution of **32** (1.00 g, 1.21 mmol) in dry DMF (11 mL), NaH (0.87 g, 21.78 mmol) was added and the mixture was stirred at 0 °C for 10 min. 1-Bromotetradecane (6.68 mL, 21.78 mmol) was added dropwise, under Ar atmosphere, and the mixture was stirred overnight at 60 °C. The reaction was quenched with MeOH (5 mL) and stirred for 10 min. Solvents were removed, and the resulting residue was suspended in DCM (50 mL). The suspension was washed with H_2O (3 × 15 mL), and the organic layer was dried ($MgSO_4$), filtered, concentrated, and purified by column chromatography (1:50 → 1:30 EtOAc−cyclohexane). Yield 77% (1.50 g, 0.93 mmol); R_f = 0.77 (1:15 EtOAc−cyclohexane); $[\alpha]_D$ = +50.2 (*c* 1.0, DCM). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.52−7.22 (m, 30 H, Ph), 5.33 (d, 2 H, $J_{1,2} = 3.6$ Hz, H-1), 4.03 (bd, 2 H, $J_{4,5} = 9.4$ Hz, H-5), 3.79 (m, 2 H, OCH_2), 3.71−3.52 (m, 8 H, OCH_2), 3.55 (t, 2 H, $J_{2,3} = J_{3,4} = 9.4$ Hz, H-3),3.44 (t, 2 H, H-4), 3.46−3.35 (m, 6 H, OCH_2, H-6a, H-2), 3.22 (m, 2 H, OCH_2), 3.12 (dd, 2 H, $J_{6a,6b} = 10.0$ Hz, $J_{5,6b} = 3.1$ Hz, H-6b), 1.76−156 (m, 12 H, CH_2), 1.28 (m, 132 H, CH_2), 0.91 (t, 18 H, $^3J_{H,H} = 6.3$ Hz, CH_3). ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 144.1, 128.8, 127.6, 126.8 (Ph), 93.7 (C-1), 86.2 (Ph_3C), 81.4 (C-3), 80.6 (C-2), 78.2 (C-4), 73.7, 73.0, 71.3 (OCH_2), 70.5(C-5), 62.1 (C-6), 31.9−22.7 (CH_2), 14.1 (CH_3). ESI MS: m/z = 2027.4 $[M + Na]^+$. Anal. Calcd for $C_{134}H_{218}O_{11}$: C, 80.26; H, 10.96. Found: C, 80.35; H, 11.05.

*2,3,4,2′,3′,4-Hexa-O-hexyl-α,α′-trehalose (**35**).* To a solution of **33** (0.68 g, 0.52 mmol) in 1:1 DCM−MeOH (25 mL), *p*-toluenesulfonic acid monohydrate (0.08 g, 0.42 mmol) was added and the solution was stirred at rt for 4 h. The mixture was diluted with DCM, washed with saturated aqueous $NaHCO_3$, dried ($MgSO_4$), filtered, and concentrated. The resulting residue was purified by column chromatography (1:8 → 1:2 EtOAc−cyclohexane). Yield 48% (0.21 g, 0.24 mmol); R_f = 0.25 (1:2 EtOAc−cyclohexane); $[\alpha]_D$ = +103.0 (*c* 1.0, DCM). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 5.06 (d, 2 H, $J_{1,2} = 3.7$ Hz, H-1), 3.90 (dt, 2 H, $J_{4,5} = 9.2$ Hz, $J_{5,6a} = J_{5,6b} = 2.9$ Hz, H-5), 3.79 (m, 4 H, OCH_2), 3.73−3.63 (m, 8 H, H-6a, H-6b, OCH_2), 3.60−3.43 (m, 4 H, OCH_2), 3.57 (t, 2 H, $J_{2,3} = J_{3,4} = 9.2$ Hz, H-3), 3.23 (t, 2 H, H-4), 3.18 (dd, 2 H, H-2), 2.03 (bs, 2 H, OH), 1.60−1.47 (m, 12 H, CH_2), 1.37−1.27 (m, 36 H, CH_2), 0.87 (m, 18 H, CH_3). ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 93.6 (C-1), 81.0 (C-3), 80.4 (C-2), 77.9 (C-4), 73.6, 73.3, 71.5 (OCH_2), 71.4 (C-5), 61.8 (C-6), 31.8−22.6 (CH_2), 14.0 (CH_3). ESI MS: m/z = 869.7 $[M + Na]^+$, 885.7 $[M + K]^+$. Anal. Calcd for $C_{48}H_{94}O_{11}$: C, 68.05; H, 11.18. Found: C, 67.89; H, 11.04.

*2,3,4,2′,3′,4-Hexa-O-tetradecyl-α,α′-trehalose (**36**).* To a solution of **34** (1.49 g, 0.74 mmol) in 1:1 DCM−MeOH (36 mL), *p*-toluenesulfonic acid monohydrate (0.11 g, 0.50 mmol) was added and the solution was stirred at rt for 4 h. The mixture was diluted with DCM, washed with saturated aqueous $NaHCO_3$, dried ($MgSO_4$), filtered, and concentrated. Purification by column chromatography (1:9 → 1:5 EtOAc−cyclohexane) of the residue afforded **37**. Yield 47% (0.53 g, 0.34 mmol); R_f = 0.20 (1:5 EtOAc−cyclohexane); $[\alpha]_D$ = +58.4 (*c* 1.0, DCM). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 5.06 (d, 2 H, $J_{1,2} = 3.7$ Hz, H-1), 3.90 (dt, 2 H, $J_{4,5} = 9.2$ Hz, $J_{5,6a} = J_{5,6b} = 2.6$ Hz, H-5), 3.84−3.64 (m, 4 H, OCH_2), 3.73−3.63 (m, 12 H, H-6a, H-6b, OCH_2), 3.58 (t, 2 H, $J_{2,3} = J_{3,4} = 9.2$ Hz, H-3), 3.24 (t, 2 H, H-4), 3.19 (dd, 2 H, H-2), 1.86 (bs, 2 H, OH), 1.61−1.49 (m, 12 H, CH_2), 1.26 (bs, 132 H, CH_2), 0.88 (t, 18 H, $^3J_{H,H} = 6.9$ Hz,CH_3). ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 93.7 (C-1), 81.1 (C-3), 80.5 (C-2), 78.0 (C-4), 73.6, 73.3, 71.6 (OCH_2), 71.1 (C-5), 61.9 (C-6), 31.9−22.7 (CH_2), 14.1 (CH_3). ESI MS: m/z = 1543.2 $[M + Na]^+$. Anal. Calcd for $C_{96}H_{190}O_{11}$: C, 75.83; H, 12.59. Found: C, 75.70; H, 12.41.

*6,6′-Dideoxy-2,3,4,2′,3′,4-hexa-O-hexyl-6,6′-diiodo-α,α′-trehalose (**37**).* To a solution of **35** (0.10 g, 0.12 mmol) in toluene (5 mL), triphenylphosphine (0.11 g, 0.43 mmol) and imidazole (0.05 g, 0.81 mmol) were added and the mixture was stirred at rt until complete dissolution. Iodine (0.11 g, 0.40 mmol) was added in portions, and the solution was vigorously stirred at 70 °C for 5 h. Satd aq $NaHCO_3$ solution (10 mL) was added, and the mixture was stirred for 5 min. Additional iodine was then added until the aqueous solution turned to a slightly brown color, then aqueous 10% $Na_2S_2O_3$ was added until complete decoloration of both organic and aqueous layer. The organic layer was then separated, dried ($MgSO_4$), filtered, concentrated, and purified by column chromatography (1:15 EtOAc−cyclohexane). Yield 94% (0.12 g, 0.12 mmol); R_f = 0.75 (1:8 EtOAc−cyclohexane); $[\alpha]_D$ = +50.1 (*c* 1.0, DCM). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 5.17 (d, 2 H, $J_{1,2} = 3.3$ Hz, H-1), 3.83 (m, 4 H, OCH_2), 3.72−3.47 (m, 12 H, H-5, H-3, OCH_2), 3.39 (m, 4 H, H-6a, H-6b), 3.24 (dd, 2 H, Hz, $J_{2,3} = 9.1$ Hz, H-2), 3.05 (t, 2 H, $J_{3,4} = J_{3,4} = 9.1$ Hz, H-4), 1.62−1.49 (m, 12 H, CH_2), 1.30 (m, 36 H, CH_2), 0.89 (m, 18 H, CH_3). ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 92.9 (C-1), 81.9 (C-4), 80.8 (C-3), 80.2 (C-2), 73.5, 73.4, 71.9 (OCH_2), 69.1 (C-5), 31.8−22.6 (CH_2), 14.0 (CH_3), 8.7 (C-6). ESI MS: m/z = 1089.6 $[M + Na]^+$. Anal. Calcd for $C_{48}H_{92}I_2O_9$: C, 54.03; H, 8.69. Found: C, 53.88; H, 8.77.

*6,6′-Dideoxy-2,3,4,2′,3′,4-hexa-O-tetradecyl-6,6′-diiodo-α,α′-trehalose (**38**).* To a solution of **36** (0.44 g, 0.29 mmol) in toluene (13 mL), triphenylphosphine (0.27 g, 1.02 mmol) and imidazole (0.07 g, 1.89 mmol) were added and the mixture was stirred at rt until complete dissolution. Iodine (0.26 g, 0.93 mmol) was added in portions, and the solution was vigorously stirred at 70 °C for 5 h. Satd $NaHCO_3$ solution (20 mL) was added, and the mixture was stirred for 5 min. Additional iodine was then added until the aqueous solution got slightly brown, and then an aqueous 10% $Na_2S_2O_3$ solution was added until complete decoloration of both the organic and aqueous layers. The organic layer was then separated, dried ($MgSO_4$), filtered, concentrated, and purified by column chromatography (1:25 EtOAc−cyclohexane). Yield 96% (0.52 g, 0.28 mmol); R_f = 0.69 (1:20 EtOAc−cyclohexane); $[\alpha]_D$ = +47.2 (*c* 1.0, DCM). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 5.17 (d, 2 H, $J_{1,2} = 3.5$ Hz, H-1), 3.83 (m, 4 H, OCH_2), 3.70−3.48 (m, 12 H, H-5, H-3, OCH_2), 3.42 (dd, 2 H, $J_{6a,6b} = 10.7$ Hz, $J_{5,6a} = 2.9$ Hz, H-6a), 3.36 (dd, 2 H, $J_{5,6a} = 5.2$ Hz, H-6b), 3.24 (dd, 2 H, Hz, $J_{2,3} = 9.4$ Hz, H-2), 3.05 (t, 2 H, $J_{3,4} = J_{3,4} = 9.4$ Hz, H-4), 1.59−1.51 (m, 12 H, CH_2), 1.26 (bs, 132 H, CH_2), 0.88 (t, 18 H, $^3J_{H,H} = 6.9$ Hz, CH_3). ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 92.9 (C-1), 81.9 (C-4), 80.8 (C-3), 80.3 (C-2), 73.6, 73.5, 71.9 (OCH_2), 69.1 (C-5), 31.9−22.7 (CH_2), 14.1 (CH_3), 8.7 (C-6). ESI MS: m/z = 1763.0 $[M + Na]^+$. Anal. Calcd for $C_{96}H_{188}I_2O_9$: C, 66.25; H, 10.89. Found: C, 66.09; H, 10.74.

*6,6′-Di-(2-tert-butoxycarbonylaminoethylthio)-2,3,4,2′,3′,4′-hexa-O-hexyl-α,α′-trehalose (**39**).* To a solution of **37** (0.11 g, 0.10 mmol) in dry DMF (12 mL), Cs_2CO_3 (0.09 g, 0.29 mmol) and *tert*-butyl *N*-(2-mercaptoethyl)carbamate (49 μL, 0.29 mmol) were added, under Ar atmosphere, and the reaction mixture was stirred at 60 °C for 24 h. The reaction mixture was concentrated, and the crude product was dissolved in DCM (20 mL) and washed with H_2O (2 × 30 mL). The organic phase was dried ($MgSO_4$), filtered, concentrated, and purified by column chromatography (1:8 → 1:6 EtOAc−cyclohexane). Yield 85%(0.10 g, 0.08 mmol); R_f = 0.26 (1:5 EtOAc−cyclohexane); $[\alpha]_D$ = +86.4 (*c* 1.0, DCM). IR: ν_{max} = 3350, 2928, 2855, 1710 cm^{-1}. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 5.12 (d, 2 H, $J_{1,2} = 3.3$ Hz, H-1), 4.99 (bs, 2 H, NHBoc), 4.01 (ddd, 2 H, $J_{4,5} = 9.2$ Hz, $J_{5,6b} = 6.3$ Hz, $J_{5,6a} = 2.7$ Hz, H-5), 3.81 (m, 6 H, H-3, OCH_2), 3.70−3.46 (m, 8 H, OCH_2), 3.58 (t, 2 H, $J_{2,3} = J_{3,4} = 9.2$ Hz, H-3), 3.29 (q, 2 H, $^3J_{H,H} = {}^3J_{H,NH} = 6.0$ Hz, CH_2N), 3.23 (dd, 2 H, H-2), 3.16 (t, 2 H, H-4), 2.82 (dd, 1 H, $J_{6a,6b} = 13.5$ Hz, H-6a), 2.72 (dd, 2 H, H-6b), 2.69 (t, 4 H, CH_2S), 1.60−1.47 (m, 12 H, CH_2), 1.43 (s, 18 H, $C(CH_3)_3$), 1.34−1.28 (m, 36 H, CH_2), 0.89 (m, 18 H, CH_3). ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 155.7 (CO), 92.3 (C-1), 81.0 (C-3), 80.5 (C-2), 80.4 (C-4), 79.2 (CMe_3), 73.4, 73.2, 71.7 (OCH_2), 71.2 (C-5), 39.8 (CH_2S), 33.8 (C-6), 33.7 (CH_2N), 31.8−30.2 (CH_2), 28.4 (CMe_3), 25.9, 25.8, 22.6 (CH_2), 14.0 (CH_3). ESI MS: m/z = 1187.9 $[M + Na]^+$, 1203.8 $[M + K]^+$. Anal. Calcd for $C_{62}H_{120}N_2O_{13}S_2$: C, 63.88; H, 10.38; N, 2.40. Found: C, 63.69; H, 10.21; N, 5.19.

*6,6′-Di-(2-tert-butoxycarbonylaminoethylthio)-2,3,4,2′,3′,4′-hexa-O-tetradecyl-α,α′-trehalose (**40**).* To a solution of **38** (0.28 g, 0.13 mmol) in dry DMF (15 mL), Cs_2CO_3 (0.12 g, 0.36 mmol) and *tert*-butyl *N*-(2-mercaptoethyl)carbamate (61 μL, 0.36 mmol) were added, under Ar atmosphere, and the reaction mixture was stirred at 60 °C for 24 h. The solvent was evaporated, and the crude product was dissolved in DCM (20 mL) and washed with H_2O (2 × 30 mL). The organic phase was separated, dried ($MgSO_4$), filtered, concentrated, and purified by column chromatography (1:8 EtOAc−cyclohexane). Yield 99% (0.24 g, 0.13 mmol); R_f = 0.53 (1:5 EtOAc−cyclohexane); $[\alpha]_D$ = +49.3 (*c* 1.0, DCM). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 5.12 (d, 2 H, $J_{1,2}$ = 3.4 Hz, H-1), 4.99 (bs, 2 H, NHBoc), 4.01 (ddd, 2 H, $J_{4,5}$= 9.6 Hz, $J_{5,6a}$ = 2.6 Hz, $J_{5,6b}$ = 6.1 Hz, H-5), 3.81 (m, 4 H, OCH_2), 3.69−3.46 (m, 8 H, OCH_2), 3.58 (t, 2 H, $J_{2,3}$ = $J_{3,4}$ = 9.6 Hz, H-3), 3.30 (bq, 2 H, $^3J_{H,H}$ = $^3J_{H,NH}$ = 6.0 Hz, CH_2N), 3.23 (dd, 2 H, H-2), 3.17 (t, 2 H, H-4), 2.83 (dd, 2 H, $J_{6a,6b}$ = 13.7 Hz, H-6a), 2.72 (dd, 2 H, H-6b), 2.69 (t, 4 H, CH_2S), 1.60−1.51 (m, 12 H, CH_2), 1.44 (s, 18 H, CMe_3), 1.26 (bs, 132 H, CH_2), 0.88 (t, 18 H, $^3J_{H,H}$ = 6.9 Hz, CH_3). ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 155.7 (CO), 92.3 (C-1), 81.0 (C-3), 80.5 (C-2), 80.4 (C-4), 79.3 (CMe_3), 73.5, 73.2, 71.6 (OCH_2), 71.2 (C-5), 39.8 (CH_2S), 33.8 (C-6), 33.7 (CH_2N), 31.9, 30.7, 30.5, 30.3, (CH_2), 29.7 (CMe_3), 29.4−22.7 (CH_2), 14.1 (CH_3). ESI MS: *m*/*z* = 1861.5 $[M + Na]^+$. Anal. Calcd for $C_{110}H_{216}N_2O_{13}S_2$: C, 71.84; H, 11.84; N, 1.52; S, 3.49. Found: C, 71.90; H, 11.72; N, 1.44; S, 3.38.

*6,6′-Di-(2-aminoethylthio)-2,3,4,2′,3′,4′-hexa-O-hexyl-α,α′-trehalose Dihydrochloride (**7**).* Treatment of **39** (0.10 g, 0.09 mmol) with 1:1 TFA−DCM (2 mL) and freeze-drying from 10:1 H_2O/0.1 N HCl solution afforded **7**. Yield quant (0.09 g, 0.09 mmol); R_f = 0.72 (10:1:1 CH_3CN−H_2O−NH_4OH); $[\alpha]_D$ = +90.3 (*c* 1.0, DCM). IR: ν_{max} = 3300, 2928, 2859, 1099 cm^{-1}. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 8.13 (bs, 6 H, NH_3^+), 5.20 (d, 2 H, $J_{1,2}$ = 3.2 Hz, H-1), 3.98 (dt, 2 H, $J_{4,5}$ = 9.5 Hz, $J_{5,6a}$ = $J_{5,6b}$ = 4.3 Hz, H-5), 3.79 (m, 4 H, OCH_2), 3.69−3.43 (m, 8 H, OCH_2), 3.56 (t, 2 H, $J_{2,3}$ = $J_{3,4}$ = 9.5 Hz, H-3), 3.25 (dd, 2 H, H-2), 3.18 (bs, 4 H, CH_2N), 3.11 (t, 2 H, H-4), 2.97 (bd, 2 H, $J_{6a,6b}$ = 14.0 Hz, H-6a), 2.82 (m, 6 H, CH_2S, H-6b), 1.59−1.50 (m, 12 H, CH_2), 1.28 (m, 36 H, CH_2), 0.88 (m, 18 H, CH_3). ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 91.9 (C-1), 80.9 (C-3), 80.2 (C-2), 80.0 (C-4), 73.5, 73.4, 71.8 (OCH_2), 71.0 (C-5), 39.4 (CH_2N), 34.4 (C-6), 31.8 (CH_2S), 31.7,30.6, 30.4, 30.2, 25.9, 25.8, 22.6, 22.5 (CH_2), 14.0 (CH_3). ESI MS: *m*/*z* = 965.9 $[M - 2Cl]^+$. Anal. Calcd for $C_{52}H_{102}N_2O_9S_2$·2HCl: C, 60.14; H, 10.29; N, 2.70; S, 6.17. Found: C, 59.86 H, 10.02; N, 2.41; S, 5.88.

*6,6′-Di-(2-aminoethylthio)-2,3,4,2′,3′,4′-hexa-O-tetradecyl-α,α′-trehalose Dihydrochloride (**8**).* Treatment of **40** (0.09 mg, 0.05 mmol) with 1:1 TFA−DCM (1 mL) and freeze-drying from 10:1 H_2O/0.1 N HCl solution afforded **8**. Yield quant (0.09 g, 0.05 mmol); R_f = 0.21 (EtOAc); $[\alpha]_D$ = +61.2 (*c* 1.0, DCM). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 6.39 (bs, 6 H, NH_3^+), 5.21 (d, 2 H, $J_{1,2}$ = 3.5 Hz, H-1), 3.97 (dt, 2 H, $J_{4,5}$ = 9.6 Hz, $J_{5,6a}$ = $J_{5,6b}$ = 4.7 Hz, H-5), 3.79 (m, 4 H, OCH_2), 3.69−3.43 (m, 8 H, OCH_2), 3.56 (t, 2 H, $J_{2,3}$ = $J_{3,4}$ = 9.6 Hz, H-3), 3.25 (dd, 2 H, H-2), 3.18 (t, 4 H, $^3J_{H,H}$ = 6.3 Hz,CH_2N), 3.11 (t, 2 H, H-4), 3.07−2.94 (m, 4 H, H-6a, H-6b), 2.82 (m, 4 H, CH_2S), 1.58−1.49 (m, 12 H, CH_2), 1.26 (bs, 132 H, CH_2), 0.88 (m, 18 H, $^3J_{H,H}$ = 7.0 Hz, CH_3). ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 91.9 (C-1), 80.9 (C-3), 80.2 (C-2), 80.0 (C-4), 73.5, 73.4, 71.8 (OCH_2), 71.0 (C-5), 39.5 (CH_2N), 34.6 (C-6), 31.9 (CH_2S), 31.9−22.7 (CH_2), 14.1 (CH_3). ESI MS: *m*/*z* = 1639.4 $[M - 2 Cl]^+$. Anal. Calcd for $C_{100}H_{200}N_2O_9S_2$·2HCl: C, 70.17; H, 11.90; N, 1.64; S, 3.75. Found: C, 69.82; H, 11.77; N, 1.39; S, 3.41.

*6,6′-Dideoxy-2,3,4,2′,3′,4′-hexa-O-hexanoyl-6,6′-diiodo-α,α′-trehalose (**42**).* To a solution of 6,6′-dideoxy-6,6′-diiodo-trehalose **41** (5.78 g, 10.30 mmol) and DMAP (6.37 g, 52.20 mmol) in dry DMF (80 mL), hexanoic anhydride (16 mL, 69.60 mmol) was added dropwise, under Ar atmosphere at 0 °C, and the reaction mixture was stirred at rt for 6 h. Then MeOH (60 mL) was added, and the mixture was stirred at rt for 2 h. The solution was poured into H_2O−ice mixture (100 mL) and extracted with DCM (50 mL). The organic phase was then washed with 2N H_2SO_4 (2 × 50 mL), H_2O (2 × 50 mL), and saturated aqueous $NaHCO_3$ (2 × 50 mL), dried ($MgSO_4$), filtered, concentrated, and purified by column chromatography (1:12 EtOAc−petroleum ether). Yield 76% (1.20 g, 7.82 mmol); R_f = 0.35 (1:6 EtOAc−petroleum ether). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 5.44 (t, 2 H, $J_{2,3}$ = $J_{3,4}$ = 9.5 Hz, H-3), 5.35 (d, 2 H, $J_{1,2}$ = 3.7 Hz, H-1), 5.1 (dd, 2 H, H-2), 4.84 (t, 2 H, $J_{4,5}$ = 9.5 Hz, H-4), 3.82 (ddd, 2 H, $J_{5,6a}$ = 2.5 Hz, H-5), 3.11 (dd, 2 H, $J_{6a,6b}$ = 11.0 Hz, H-6a), 2.97 (dd, 2 H, H-6b), 2.22 (m, 12 H, H-2_{Hex}), 1.58 (m, 12 H, H-3_{Hex}), 1.17 (m, 24 H, H-4_{Hex}, H-5_{Hex}), 0.84 (m, 18 H, H-6_{Hex}). ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 172.6, 172.5, 172.4 (CO), 91.5 (C-1), 72.0 (C-4), 70.0 (C-5), 69.3 (C-3), 69.2 (C-2), 34 (C-2_{Hex}), 31.2 (C-4_{Hex}), 24.4 (C-3_{Hex}), 22.2 (C-5_{Hex}), 14.1 (C-6_{Hex}), 2.6 (C-6_{Hex}). ESI MS: *m*/*z* = 1173.4 $[M + Na]^+$. Anal. Calcd for $C_{47}H_{78}I_2O_{15}$: C, 49.65; H, 6.92. Found: C, 50.12; H, 7.01.

*2,3,4,2′,3′,4′-Hexa-O-hexanoyl-6,6′-bis(2-tert-butoxycarbonylaminoethylthio)-α,α′-trehalose (**43**).* To a solution of **42** (0.19 g, 0.17 mmol) in dry DMF (1.5 mL), Cs_2CO_3 (0.15 g, 0.47 mmol) and *tert*-butyl *N*-(2-mercaptoethyl)carbamate (80 μL, 0.47 mmol) were added, under Ar atmosphere, and the reaction mixture was stirred at 60 °C for 24 h. The reaction mixture was concentrated, and the crude product was dissolved in DCM (10 mL) and washed with water (2 × 20 mL). The organic phase was dried ($MgSO_4$), filtered, and concentrated. The residue was purified by column chromatography (1:3 EtOAc−cyclohexane). Yield 58% (0.12 mg, 0.10 mmol); R_f = 0.52 (1:2 EtOAc−cyclohexane); $[\alpha]_D$ = +84.5 (*c* 1.0, DCM). IR: ν_{max} = 2959, 1749, 1709 cm^{-1}. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 5.43 (t, 2 H, $J_{3,2}$ = $J_{3,4}$ = 9.5 Hz, H-3), 5.25 (d, 1 H, $J_{1,2}$ = 4.1 Hz, H-1), 5.00 (dd, 1 H, H-2), 4.94 (t, 2 H, H-4), 3.88 (m, 2 H, H-5), 3.2 (s, 4 H, CH_2N), 2.61 (m, 4 H, CH_2S), 2.52 (m, 4 H, H-6a, H-6b), 2.23 (m, 12 H, H-2_{Hex}), 1.50 (m, 6 H, H-3_{Hex}), 1.41 (s, 18 H, CMe_3), 1.23 (m, 24 H, H-4_{Hex}, H-5_{Hex}), 0.83 (t, 18 H, H-6_{Hex}). ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 172.6, 172.5, 172.4 (C-1_{Hex}), 155.7 (CO carbamate), 91.4 (C-1), 71.2 (C-4), 71.1 (C-5), 69.6 (C-2, C-3), 39.7 (CH_2N), 34.1, 34.0 (C-2_{Hex}), 33.8 (CH_2S), 24.4 (C-3_{Hex}), 28.4 (CMe_3), 22.3 (C-4_{Hex}, C-5_{Hex}), 13.8 (C-6_{Hex}). ESI MS: *m*/*z* = 1271.8 $[M + Na]^+$. Anal. Calcd for $C_{62}H_{108}N_2O_{19}S_2$: C, 59.59; H, 8.71; N, 2.24; S, 5.13. Found: C, 59.67; H, 8.69; N, 2.32; S, 4.89.

*2,3,4,2′,3′,4′-Hexa-O-hexanoyl-6,6′-bis(2-tert-aminoethylthio)-α,α′-trehalose dihydrochloride (**9**).* Treatment of **43** (0.07 g, 0.04 mmol) with 1:1 TFA−DCM (2 mL) and freeze-drying from 10:1 H_2O/0.1 N HCl solution afforded **9**. Yield quant (0.07 g, 0.04 mmol); $[\alpha]_D$ = +66.7 (*c* 0.9, EtOAc). IR: ν_{max} = 2957, 1740, 1686 cm^{-1}. ^{1}H NMR (300 MHz, DMSO-d_6): δ = 5.35 (t, $J_{2,3}$ = 9.7 Hz, 2 H, H-3), 5.29 (d, 2 H, $J_{1,2}$ = 4.0 Hz, H-1), 5.08 (m, 2 H, H-2, H-3), 3.95 (m, 2 H, H-5), 2.95 (bs, 4 H, CH_2N), 2.69 (t, 4 H, $J_{H,H}$ = 7.0 Hz, CH_2S), 2.67 (bd, 4 H, H-6a, H-6b), 2.27 (m, 12 H, H-2_{Hex}), 1.49 (m, 12 H, H-3_{Hex}), 1.24 (m, 24 H, H-4_{Hex}, H-5_{Hex}), 0.85 (t, 18 H, H-6_{Hex}). ^{13}C NMR (75.5 MHz, DMSO-d_6): δ = 171.8, 171.7 (CO), 90.9 (C-1), 70.9 (C-5), 70.0, 69.2, (C-2, C-4), 69.4 (C-3), 39.2 (CH_2N), 31.5 (C-6), 33.9 (C-2_{Hex}), 30.3 (CH_2S), 23.9 (C-3_{Hex}), 30.6 (C-4_{Hex}), 21.8 (C-5_{Hex}), 13.3 (C-6_{Hex}). ESI MS: *m*/*z* = 1049.6 $[M]^+$. Anal. Calcd for $C_{52}H_{94}Cl_2N_2O_{15}S_2$: C, 55.65; H, 8.44; N, 2.50; S, 5.71. Found: C, 55.29; H, 8.18; N, 2.14; S, 5.33

*6,6′-Diazido-6,6′-dideoxy-2,3,4,2′,3′,4′-hexa-O-hexanoyl-α,α′-trehalose (**45**).* To a solution of **42** (1.58 g, 1.37 mmol) in dry DMF (8 mL), NaN_3 (0.25 g, 3.60 mmol) was added. The reaction mixture was stirred overnight at 40 °C under Ar atmosphere. The mixture was poured into H_2O−ice mixture (20 mL), and the product was extracted with DCM (4 × 20 mL). The organic phase was dried ($MgSO_4$), filtered, and concentrated. The residue was purified by column chromatography (1:12 EtOAc−cyclohexane) to afford **45**. Yield 72% (0.91 g, 0.98 mmol); R_f = 0.53 (1:6 EtOAc−cyclohexane); $[\alpha]_D$ = +108.7 (*c* 1.0, DCM). IR: ν_{max} = 2958, 2104, 1750, 735 cm^{-1}. ^{1}H NMR (300 MHz, CD_3OD): δ = 5.52 (t, 2 H, $J_{2,3}$ = $J_{3,4}$ = 9.8 Hz, H-3), 5.41 (d, 2 H, $J_{1,2}$ = 4.0 Hz, H-1), 5.13 (dd, 2 H, H-2), 5.09 (t, 2 H, $J_{4,5}$ = 9.8 Hz, H-4), 4.08 (ddd, 2 H, $J_{5,6a}$ = 7.0 Hz, $J_{5,6b}$ = 2.6 Hz, H-5), 3.44 (dd, 2 H, $J_{6a,6b}$ = 13.0 Hz, H-6a), 2.97 (dd, 2 H, H-6b), 2.33 (m, 12 H, H-2_{Hex}), 1.58 (m, 12 H, H-3_{Hex}), 1.33 (m, 24 H, H-4_{Hex},H-5_{Hex}), 0.91 (m, 18 H, H-6_{Hex}). ^{13}C NMR (75.5 MHz, CD_3OD): δ = 175.2−174.2 (CO), 93.7 (C-1), 71.5 (C-3), 71.3 (C-2), 71.6 (C-5), 71 (C-4), 52.2 (C-6), 34.9 (C-2_{Hex}), 33.2 (C-4_{Hex}), 25.8 (C-3_{Hex}), 23.6 (C-5_{Hex}), 14.8

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(C-6_{Hex}). ESI MS: m/z = 1003.5 $[M + Na]^+$. Anal. Calcd for $C_{25}H_{43}N_8O_{15}$: C, 58.76; H, 8.22; N, 8.57. Found: C, 58.84; H, 8.32; N, 8.60.

*6,6′-Di-(4-tert-butoxycarbonylaminomethyl-1H-1,2,3-triazol-1-yl)-6,6′-dideoxy-2,3,4,2′,3′,4′-hexa-O-hexanoyl-α,α′-trehalose (**46**).* To a solution of **44** (0.25 g, 0.25 mmol) and **45** (0.08 g, 0.56 mmol) in H_2O-^{t}BuOH 9:1 (15 mL), the Cu-supported catalyst Si-BPA·Cu^+ (0.02 g) was added and the reaction mixture was refluxed for 36 h at 85 °C. The catalyst was filtered, and the solvent was concentrated. The residue was purified by column chromatography (1:1 → 2:1 EtOAc–cyclohexane). Yield quant (0.33 g, 0.25 mmol); R_f = 0.73 (2:1 EtOAc–cyclohexane); $[\alpha]_D$ = +56.7 (*c* 1.0, DCM). IR: ν_{max} = 2957, 1752, 1714, 735 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$): δ = 7.52 (s, 2 H, ═CH), 5.45 (t, 2 H, $J_{2,3} = J_{3,4}$ = 9.8 Hz, H-3), 5.30 (bs, 2 H, N*H*Boc), 4.97 (dd, 2 H, $J_{1,2}$= 4.0 Hz, H-2), 4.87 (t, 2 H, $J_{4,5}$ = 9.8 Hz, H-4), 4.83 (d, 2 H, H-1), 4.48 (bd, 2 H, $J_{6a,6b}$ = 13.7 Hz, H-6a), 4.37 (d, 4 H, $J_{H,H}$ = 6.0 Hz, CH_2 triazole), 4.12 (dd, 2 H, $J_{5,6b}$ = 8.8 Hz, H-6b), 4.07 (m, 2 H, H-5), 2.25 (m, 12 H, H-2_{Hex}), 1.55 (m, 12 H, H-3_{Hex}), 1.46 (s, 18 H, CMe_3), 1.29 (m, 24 H, H-4_{Hex}, H-5_{Hex}), 0.89 (m, 18 H, H-6_{Hex}). ^{13}C NMR (75.5 MHz, CD_3OD): δ = 173.5–173.2 (CO ester), 155.5 (CO carbamate), 146.0 (C-4 triazole), 122.3 (C-5 triazole), 91.7 (C-1), 79.5 (CMe_3), 69.9 (C-3, C-5), 69.8 (C-4), 69.3 (C-2), 50.7 (C-6), 36.4 (CH_2-triazole), 34.3 (C-2_{Hex}), 31.9 (C-4_{Hex}), 28.7 (CMe_3) 24.6 (C-3_{Hex}), 22.6 (C-5_{Hex}), 14.1 (C-6_{Hex}). ESI MS: m/z = 1313.4 $[M + Na]^+$. Anal. Calcd for $C_{64}H_{106}N_8O_{19}$: C, 59.52; H, 8.27; N, 8.68. Found: C, 59.61; H, 8.33; N, 8.84.

*6,6′-Di-(4-Aminomethyl-1H-1,2,3-triazol-1-yl)-6,6′-dideoxy-2,3,4,2′,3′,4′-hexa-O-hexanoyl)-α,α′-trehalose Dihydrochloride (**47**).* Treatment of **46** (0.30 g, 0.23 mmol) with 1:1 TFA–DCM (4 mL) and freeze-drying from 10:1 H_2O/0.1 N HCl solution afforded **47**. Yield 97% (0.26 g, 0.22 mmol); $[\alpha]_D$ = +48.9 (*c* 1.0, MeOH). IR: ν_{max} = 2956, 1751, 1464, 1026 cm^{-1}. ^1H NMR (300 MHz, CD_3OD): δ = 8.05 (s, 2 H, CH triazole), 5.49 (t, 2 H, $J_{3,4} = J_{2,3}$ = 9.8 Hz, H-3), 5.06 (dd, 2 H, $J_{1,2}$ = 4.0 Hz, H-2), 4.96 (d, 2 H, $J_{1,2}$ = 3.6 Hz, H-1), 4.95 (t, 2 H, $J_{4,5}$ = 9.8 Hz, H-4), 4.63 (dd, 2 H, $J_{5,6a}$ = 2.6 Hz, $J_{5,6b}$ = 14.6 Hz, H-6), 4.53 (dd, 2 H, $J_{5,6b}$ = 7.9 Hz, H-6b), 4.27 (bs, 4 H, CH_2NH_2), 4.24 (m, 1 H, H-5), 2.32 (m, 12 H, H-2_{Hex}), 1.59 (m, 12 H, H-3_{Hex}), 1.33 (m, 24 H, H-4_{Hex},H-5_{Hex}), 0.93 (m, 18 H, H-6_{Hex}). ^{13}C NMR (75.5 MHz, CD_3OD): δ = 174.5–173.6 (CO), 141.5 (C-4 triazole) 127.1 (C-5 triazole), 92.7 (C-1), 71.2 (C-2), 70.6 (C-3), 70.4 (C-4), 70.3 (C-5), 51.5 (C-6), 35.2 (CH_2NH_2), 34.8 (C-2_{Hex}), 32.1 (C-4_{Hex}), 25.8 (C-3_{Hex}), 22.6 (C-5_{Hex}), 14.2 (C-6_{Hex}). ESI MS: m/z = 1091.4 $[M]^+$. Anal. Calcd for $C_{54}H_{92}N_8O_{15}$: C, 55.71; H, 7.96; N, 9.62. Found: C, 55.48; H, 7.77; N, 9.97.

*6,6′-Di-[4-(2-N′-(2-(N-tert-butoxycarbonyl)aminoethyl)-thioureido))methyl-1H-1,2,3-triazolyl-]2,3,4,2′,3′,4′-hexa-O-hexanoyl)-6,6′-dideoxy-α,α′-trehalose (**48**).* To a solution of **47** (0.14 g, 0.12 mmol) and Et_3N (37 μL, 0.36 mmol) in DCM (10 mL), *tert*-butyl *N*-(2-isothiocyanoethyl) carbamate (0.07 g, 0.36 mmol) was added and the reaction mixture was stirred overnight at rt. The reaction mixture was washed with aqueous diluted HCl (3 × 10 mL), and the organic phase was dried ($MgSO_4$), filtered, and concentrated. The residue was purified by column chromatography (2:1 → 3:1 EtOAc–cyclohexane). Yield 67% (0.12 g, 0.08 mmol); R_f = 0.40 (3:1 EtOAc–cyclohexane); $[\alpha]_D$ = +59.8 (*c* 1.0, DCM). UV (DCM): 249 nm (ε_{mM} 53.9). IR: ν_{max} = 2959, 1752, 1701, cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$): δ = 7.80 (s, 2 H, CH triazole), 7.07 (bs, 4 H, NHCS), 5.39 (t, 2 H, $J_{3,4} = J_{2,3}$ = 9.7 Hz, H-3), 5.29 (bs, 2 H, N*H*Boc), 4.98–4.76 (m, 4 H, H-1, H-4, CH_2 triazole), 4.62 (dd, 2 H, H-2), 4.47 (d, 2 H, $J_{6a,6b}$ = 14.0 Hz, H-6a), 4.28 (dd, 2 H, $J_{5,6b}$ = 8.0 Hz, H-6b), 3.97 (bt, 2 H, H-5), 3.60 (bs, 4 H, CH_2CH_2NHBoc), 3.31 (m, 4 H, $^3J_{H,H}$ = 5.4 Hz, CH_2NHBoc), 2.22 (m, 12 H, H-2_{Hex}), 1.53 (m, 12 H, H-3_{Hex}), 1.39 (s, 18 H, CMe_3), 1.27 (m, 24 H, H-4_{Hex}, H-5_{Hex}), 0.86 (m, 18 H, H-6_{Hex}). ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 173.2–172.5 (CO ester), 156.2 (CO carbamate), 141.5 (C-4 triazole), 124.3 (C-5 triazole), 96.7 (C-1), 79.7 (CMe_3), 69.6–68.7 (C-2, C-3, C-4, C-5), 50.3 (C-6), 40.0 (CH_2CH_2NHBoc), 39.5 (CH_2NHBoc), 33.9 (C-2_{Hex}), 31.2 (C-4_{Hex}), 28.3 (CMe_3), 24.3 (C-3_{Hex}), 22.2 (C-5_{Hex}), 13.1 (C-6_{Hex}). ESI MS: m/z = 1517.4 $[M + Na]^+$. Anal. Calcd for $C_{70}H_{118}N_{12}O_{19}S_2$: C, 56.20; H, 7.95; N, 11.24; O, 20.32; S, 4.29. Found: C, 55.95; H, 7.84; N, 11.04; S, 11.04

*[(6,6′-Di-4-(2-N′-(2-aminoethyl)thioureido))methyl-1H-1,2,3-triazol-1-yl)-6,6′-dideoxy-2,3,4,2′,3′,4′-hexa-O-hexanoyl]-α,α′-trehalose Dihydrochloride (**10**).* Treatment of **48** (0.11 g, 0.07 mmol) with TFA–DCM (2 mL) and freeze-drying from 0.1 N HCl solution afforded **10**. Yield quant (0.10 g, 0.07 mmol); $[\alpha]_D$ = +38.2 (*c* 1.0, MeOH). UV (MeOH): 243 nm (ε_{mM} 16.9). IR: ν_{max} = 2956, 2862, 1752, 1675 721 cm^{-1}. ^1H NMR (300 MHz, CD_3OD): δ = 7.92 (s, 2 H, CH triazole), 5.44 (t, 2 H, $J_{2,3} = J_{3,4}$ = 9.7 Hz, H-3), 5.08 (dd, 2 H, $J_{1,2}$ = 4.2 Hz, H-2), 4.95 (t, 2 H, H-4), 4.82 (m, 4 H, H-4, H-1, CH_2 triazole), 4.62 (dd, 2 H, $J_{6a,6b}$ = 14.0 Hz, $J_{5,6a}$ = 2.5 Hz, H-6a), 4.48 (dd, 2 H, $J_{5,6b}$ = 8.2 Hz, H-6b), 4.19 (ddd, 2 H, H-5), 3.91 (m, 4 H, $CH_2CH_2NH_2$), 3.21 (dt, 4 H, $J_{H,H}$ = 5.8 Hz, CH_2NH_2), 2.31 (m, 12 H, H-2_{Hex}), 1.59 (m, 12 H, H-3_{Hex}), 1.32 (m, 24 H, H-4_{Hex}, H-5_{Hex}), 0.93 (m, 18 H, H-6_{Hex}). ^{13}C NMR (75.5 MHz, CD_3OD): δ = 182.6 (CS), 176.0–174.5 (CO), 146.1 (C-4 triazole), 125.6 (C-5 triazole), 92.4 (C-1), 71.4 (C-4), 70.6 (C-3), 70.4 (C-5), 70.3 (C-2), 51.5 (C-6), 42.6 ($CH_2CH_2NH_2$), 40.9 (CH_2NH_2), 40.2 (CH_2–triazole), 35.0–34.8 (C-2_{Hex}), 32.5–32.4 (C-4_{Hex}), 25.7–25.4 (C-3_{Hex}), 23.2 (C-5_{Hex}), 14.3 (C-6_{Hex}). ESI MS: m/z = 1357 $[M + Cu]^+$; 711 $[M + Cu]^{2+}$. Anal. Calcd for $C_{60}H_{104}N_{12}O_{15}S_2$: C, 52.66; H, 7.66; N, 12.28; S, 4.69. Found: C, 52.71; H, 7.50; N, 12.55, 4.69.

*6,6′[4-(2,2-Bis-tert-butoxycarbonylamino)ethylaminomethyl)-1H-1,2,3-triazol-1-yl)-6-deoxy-2,3-di-O-hexanoyl]-α,α′-trehalose (**49**).* To a solution of **44** (0.31 g, 0.31 mmol) and **29** (0.24 g, 0.73 mmol) in H_2O–^{t}BuOH 9:1(15 mL), the Cu-supported catalyst Si-BPA·Cu^+ (0.02 g) was added and the reaction mixture was refluxed for 36 h at 85 °C. The catalyst was filtered, and the solvent was removed. The residue was purified by column chromatography (2:1 → 3:1 EtOAc–cyclohexane). Yield 91% (0.54 g, 0.29 mmol); R_f = 0.36 (3:1 EtOAc–cyclohexane); $[\alpha]_D$ = +57.0 (*c* 1.0, DCM). IR: ν_{max} = 2958, 2359, 2341, 1751, 1700 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$): δ = 7.57 (s, 2 H, ═C*H*), 5.47 (t, 2 H, $J_{2,3} = J_{3,4}$ = 9.8 Hz, H-3), 5.20 (bs, 4 H, NHBoc), 4.93 (dd, 2 H, $J_{1,2}$ = 3.8 Hz, H-2), 4.86 (t, 2 H, $J_{4,5}$ = 10.0 Hz, H-4), 4.77 (d, 2 H, H-1), 4.46 (bd, 2 H, $J_{6a,6b}$ = 14.0 Hz, H-6a), 4.25 (dd, 2 H, $J_{5,6b}$ = 8.7 Hz, H-6b), 4.12 (m, 2 H, H-5), 3.83 (bs, 4 H, CH_2 triazole), 3.19 (bd, 8 H, $^3J_{H,H}$ = 6.0 Hz, CH_2NHBoc), 2.55 (t, 8 H, $J_{H,H}$ = 6.0 Hz, CH_2CH_2NHBoc), 2.27 (m, 12 H, H-2_{Hex}), 1.58 (s, 36 H, CMe_3), 1.44 (m, 12 H, H-3_{Hex}), 1.30 (m, 24 H, H-4_{Hex}, H-5_{Hex}), 0.89 (m, 18 H, H-6_{Hex}). ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 174.2–173.0 (CO ester), 156.2 (CO carbamate), 143.9 (C-4 triazole), 124.3 (C-5 triazole), 91.4 (C-1), 79.1 (CMe_3), 69.5 (C-4), 69.3 (C-3), 69.2 (C-5), 68.8 (C-2), 53.1 (CH_2CH_2NHBoc), 50.6 (C-6), 48.0 (CH_2–triazole), 38.4 (CH_2NHBoc), 34.1 (C-2_{Hex}), 31.7 (C-4_{Hex}), 28.5 (CMe_3), 24.6 (C-3_{Hex}), 22.6 (C-5_{Hex}), 13.8 (C-6_{Hex}). ESI MS: m/z = 1686.6 $[M + Na]^+$. Anal. Calcd for $C_{82}H_{142}N_{12}O_{23}$: C, 59.18; H, 8.60; N, 10.10. Found: C, 59.20; H, 8.51; N, 10.15.

*6,6′[4-(2,2-Diaminoethylaminomethyl)-1H-1,2,3-triazol-1-yl)-6,6′-dideoxy-2,3,4,2′,3′,4′-tri-O-hexanoyl]-α,α′-trehalose Tetrahydrochloride (**11**).* Treatment of **49** (0.56 g, 0.34 mmol) with TFA–DCM 1:1 (6 mL) and freeze-drying from 0.1 N HCl solution afforded **11**. Yield quant (0.48 g, 0.33 mmol); $[\alpha]_D$ = +22.1 (*c* 1.0, MeOH). IR: ν_{max} = 2956, 2356, 1753, 1676, 721 cm^{-1}. ^1H NMR (300 MHz, CD_3OD): δ = 8.33 (s, 2 H, CH triazole), 5.51 (t, 2 H, $J_{3,4} = J_{2,3}$ = 9.7 Hz, H-3), 5.08 (dd, 2 H, $J_{1,2}$ = 3.9 Hz, H-2), 5.01 (t, 2 H, $J_{4,5}$ = 9.7 Hz, H-4), 4.95 (d, 2 H, H-1), 4.65 (dd, 2 H, $J_{5,6a}$ = 3.0 Hz, $J_{6a,6b}$ = 14.5 Hz, H-6a), 4.56 (dd, 2 H, $J_{5,6b}$ = 8.1 Hz, H-6b), 4.28 (bs, 4 H, CH_2 triazole), 4.18 (ddd, 2 H, H-5), 3.41 (bt, 8 H, $^3J_{H,H} = {}^3J_{H,NH}$ = 6.0 Hz, CH_2NH_2), 3.22 (bt, 8 H, $CH_2CH_2NH_2$), 2.31 (m, 12 H, H-2_{Hex}), 1.59 (m, 12 H, H-3_{Hex}), 1.33 (m, 24 H, H-4_{Hex}, H-5_{Hex}), 0.93 (m, 18 H, H-6_{Hex}). ^{13}C NMR (75.5 MHz, CD_3OD): δ = 174.2–173.2 (CO), 141.1 (C-4 triazole), 128.8 (C-5 triazole), 91.9 (C-1), 71.0 (C-4), 70.6 (C-3), 70.5 (C-5), 70.4 (C-2), 51.8 (C-6), 51.6 ($CH_2CH_2NH_2$), 47.9 (CH_2–triazole), 37.2 (CH_2NH_2), 35.1–34.8 (C-2_{Hex}), 32.5–32.4 (C-4_{Hex}), 25.6–25.5 (C-3_{Hex}), 23.4 (C-5_{Hex}), 14.3 (C-6_{Hex}). ESI MS: m/z = 1263.5 $[M]^+$; 632.0 $[M]^{2+}$. Anal. Calcd for $C_{62}H_{114}N_{12}O_{15}$: C, 52.83; H, 8.15; N, 11.93. Found: C, 52.87; H, 8.04; N, 11.74.

Determination of CMC via Pyrene Fluorescence Measurements. To assess the amphiphilicity, the critical micelle concen-

trations (CMC) of all derivatives have been determined using an established fluorescence technique based on pyrene.[46] This extremely hydrophobic dye is preferentially incorporated in the interior of micelles. The onset of micelle formation can be observed in a shift of the fluorescence excitation spectra of the samples at an emission wavelength of 372 nm. In the concentration range of aqueous micellar solutions, a shift of the excitation band in the 335 nm region toward higher wavelengths confirms the incorporation of pyrene in the hydrophobic interior of micelles. The ratio of the fluorescence intensities at 339 and 335 nm was used to quantify the shift of the broad excitation band. The critical micelle concentrations were determined from the crossover point in the low concentration range. Fluorescence spectra were recorded with an F-2500 Hitachi spectrofluorophotometer and conventional 1 cm quartz cuvettes at 37 ± 0.1 °C, using 2.5 mm excitation and emission slits.

Synthesis of Dodecanethiol Coated Gold Nanoparticles (DDT-Au NPs). A solution of tetrachloroaurate acid in milli-Q water (25 mL, 0.03 M) was mixed with a solution of tetraoctylammonium bromide in toluene (80 mL, 0.05 M). The two phases mixture was vigorously stirred until all the tetrachloroaurate was transferred into the organic layer, and the aqueous layer was discarded. To the solution was added dropwise a $NaBH_4$ aqueous solution (25 mL, 0.35 M) for 1 min, then the mixture was stirred for 1 h. The biphasic system was washed with 0.01 M HCl (1 × 25 mL), 0.01 m NaOH (1 × 25 mL), and H_2O milli-Q (3 × 25 mL). Aqueous layers were discarded, and the organic phase was stirred overnight at rt. Dodecanethiol (10 mL, 42 mmol) was added, and the mixture was refluxed for 3 h. The system was cooled to rt and spin-dried at 2000 rpm for 10 min. The supernatant was recovered, and MeOH was added to reach 1:1 mixture to precipitate the NPs and eliminate the excess of dodecanethiol, and the system was spin-dried for 10 min at 2000 rpm. Then supernatant was discarded, and the precipitate was suspended in 1 mL of $CHCl_3$.

The final concentration of DDT-Au NPs was determined by UV spectrometry. A small aliquot of NPs was 1000-fold diluted and absorbance was measured using a $\varepsilon = 8.63 \times 10^6\ M^{-1}\ cm^{-1}$.[50]

Coating of DDT-Au_NPs with Compound 11. To a solution of DDT-Au NP (120 μL) in $CHCl_3$ (30 mL) was added a solution of compound **11** in MeOH (1 mL, 2 mM). The mixture was concentrated, and milli-Q water (300 μL) was added before 1 min sonication. The dark-red solution was spin-dried in a ultrafiltration device with a poly(ether sulfone) membrane (Corning Spin-X UF) for 5 min at 6000 rpm. The precipitate was recovered by addition of milli-Q water (500 μL). Final concentration was determined measuring the absorbance at 450 nm using an $\varepsilon = 3.07 \times 10^7\ M^{-1}\ cm^{-1}$.

Biological Assays. *Reagents and Cell Cultures.* Expression plasmid for mouse MD-2 was a gift from Dr. Y. Nagai (University of Tokyo, Japan). Expression plasmid for mouse TLR4 was purchased from InvivoGen (CA, USA). Expression plasmids containing sequences of human TLR4 and MD-2 as well as the pELAM-1 firefly luciferase plasmid were a gift from Dr. C. Kirschning (Technical University of Munich, Germany). The Renilla luciferase phRL-TK plasmid was purchased from Promega (WI, USA).

The human embryonic kidney (HEK) 293 cells were provided by Dr. J. Chow (Eisai Research Institute, Andover, MA, USA). HEK293 cells were grown in DMEM supplemented with 10% FBS. Compounds were dissolved in 100% DMSO to provide 4 mM stock solutions; further working dilutions were prepared immediately before stimulation with cell medium (DMEM supplemented with 10% FBS).

Cell Activation Assay: NF-κB-Luciferase Reporter Assay. HEK 293 cells were seeded in 96-well Costar plates (Corning, NY, USA) at 1.6 × 10^4 cells/well and incubated overnight in a humidified atmosphere (5% CO_2) at 37 °C. The next day, when cells were 40−60% confluent, they were cotransfected with MD-2 (10 ng), NF-κB-dependent luciferase (70 ng), and constitutive Renilla (15 ng) reporter plasmids and TLR4 plasmid (1 ng) using PEI (7.5 molar polyethylenimine pH 7.5, Polysciences) transfection reagent. Cells were stimulated 4 h after transfection with the synthetic compounds, then 1 h later with LPS (5 nM) that was extensively vortexed immediately prior to stimulation. Cells were lysed after 16 h of stimulation in 1× reporter assay lysis buffer (Promega, USA) and analyzed for reporter gene activities using a dual-luciferase reporter assay system. Relative luciferase activity (RLA) was calculated by normalizing each sample's firefly luciferase activity for constitutive Renilla activity measured within the same sample. When plotting the data, the value of the wild type MD-2·TLR4 sample stimulated with LPS was normalized to 100 and other values were adjusted accordingly.

HEK-Blue Assay. HEK-Blue-TLR4 cells (InvivoGen) were cultured according to manufacturer's instructions. Briefly, cells were cultured in DMEM high glucose medium supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 1× Normocin (InvivoGen), 1× HEK-Blue Selection (InvivoGen). Cells were detached by the use of a cell scraper, and the cell concentration was estimated by using Trypan Blue (Sigma-Aldrich). The cells were diluted in DMEM high glucose medium supplemented as described before and seeded in multiwell plate at a density of 2 × 10^4 cells/well in 200 μL. After overnight incubation (37 °C, 5% CO_2, 95% humidity), supernatant was removed, and cell monolayers were washed with warm PBS without Ca^{2+} and Mg^{2+} and treated with increasing concentrations of compounds dissolved in DMSO−ethanol (1:1). After 30 min, the cells were stimulated with 10 nM LPS from *E. coli* O55:B5 (Sigma-Aldrich) and incubated overnight at 37 °C, 5% CO_2, and 95% humidity. As a control, the cells were treated with or without LPS (10 nM) alone. Then the supernatants were collected, and 50 μL of each sample was added to 100 μL PBS, pH 8, 0.84 mM *para*-nitrophenylphosphate (pNPP) for a final concentration of 0.8 mM pNPP. Plates were incubated for 2−4 h in the dark at rt, and then the plate reading was assessed by using a spectrophotometer at 405 nm (LT 4000, Labtech). The results were normalized with positive control (LPS alone) and expressed as the mean of percentage ± SD of at least three independent experiments.

MTT Cell Viability Assay. HEK-Blue cells were seeded in 100 μL of DMEM without Phenol Red at a density of 2 × 10^4 cells per well. After overnight incubation, 10 μL of compounds were added and the plates were incubated overnight at 37 °C, 5% CO_2, 95% humidity. DMSO and PBS were included as control. Then 10 μL of MTT solution (5 mg/mL in PBS) were added to each well. After 3 h incubation (37 °C, 5% CO_2, 95% humidity), HCl 0.1 N in 2-propanol was added (100 μL/well) to dissolve formazan crystals. Formazan concentration in the wells was determined by measuring the absorbance at 570 nm (LT 4000, Labtech). The results were normalized with untreated control (PBS) and expressed as the mean of percentage ± SD of three independent experiments.

In Vivo Endotoxin Inhibition. C57BL/6J mice (11−13 weeks old) were randomly assigned into groups and injected intraperitoneally with vehicle control (5% DMSO in PBS) (groups none and LPS only) or the inhibitory compound (2 × 10^{-7} mol compound/mouse for compounds **5**−**11**, all in 5% DMSO solution). One hour later, the mice were injected intraperitoneally with vehicle control (PBS) (group none) or with LPS from *E. coli* 055:B5 (1 × 10^{-9} mol/mouse ≈ 10 μg LPS/mouse). Three hours later, the blood was collected. Serum was tested with the mouse TNF-α ELISA kit ("ReadySetGo", eBioscience) to determine the levels of mouse TNF-α. The experiment was performed according to the manufacturer's instructions.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Activity of compounds **5**, **9**, and **10** on bone marrow-derived murine macrophages (BMDM); MTT cell toxicity tests for synthetic molecules. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

BMDM, bone marrow derived machophages; CNS, central nervous system; DC, dendritic cell; DCC, dicyclohexylcarbodiimide; DMEM, Dulbecco's Modified Eagle's Medium; DMAP, 4-dimethylaminopyridine; DMSO, dimethysulfoxide; HEK, human embryonic kidney; PTSA, *p*-toluenesulfonic acid; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide test; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; TPP, triphenylphosphine; TRAM, TRIF-related adaptor molecule; TRIF, TIR-domain-containing adapter-inducing interferon-β

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Resumen / Summary

Resumen.

En esta Tesis hemos preparado derivados de β-ciclodextrina, metil α-D-glucopiranosa y α,α'-trehalosa adaptados para su aplicación al transporte vectorizado de fármacos, la transfección génica y/o en la modulación del sistema inmune innato. Para el transporte vectorizado de fármacos se ha utilizado una estrategia de síntesis convergente en la que a un precursor monofuncionalizado de β-ciclodextrina, que actúa como plataforma de complejación de fármacos poco solubles en agua, se acopla una antena de biorreconocimiento que facilita el transporte dirigido del fármaco; en este caso chaperonas farmacológicas con estructura de iminoazúcares sp^2.

Para la preparación de vectores de transfección génica se han utilizado como plataformas sacarídicas el metil α-D-glucopiranósido y la α,α'-trehalosa, a las que se han incorporado elementos estructurales que promueven la autoorganización mediante métodos de funcionalización selectiva. De esta manera se han obtenido derivados catiónicos mono- y disacarídicos de naturaleza anfifílica capaces de formar nanopartículas, bien de manera espontánea, bien en presencia de un plásmido. La versatilidad de esta aproximación sintética permite acceder a una colección de moléculas con gran variabilidad estructural sobre las que se han realizado estudios para establecer relaciones entre su estructura molecular y sus propiedades de autoorganización y transporte de genes.

Los derivados catiónicos de la metil α-D-glucopiranosa y la α,α'-trehalosa, sintetizados mediante los métodos de funcionalización selectiva antes mencionados, presentan semejanza estructural con moléculas anfifílicas cargadas que tienen un importante papel modulador de la actividad del sistema inmune innato al interaccionar con el receptor "toll-like" TLR4. Dado que tanto derivados catiónicos como aniónicos son susceptibles de actuar como ligandos regulardores de la actividad del TLR4, hemos

puesto a punto también la síntesis de análogos aniónicos anfifílicos y, en colaboración con el Prof. F. Peri (Universidad de Milano Biccoca) hemos evaluado la actividad moduladora de la respuesta inmune de ambas familias de glicolípidos.

CICLODEXTRINAS CON ANTENAS BIORRECONOCIBLES PARA EL TRANSPORTE SELECTIVO DE CHAPERONAS FARMACOLÓGICAS

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El diseño de sistemas de encapsulación y liberación controlada de principios activos dirigido a receptores celulares específicos es crucial para la implantación de nuevas terapias que mejoren la biodisponibilidad de fármacos insolubles o biodegradables y eviten efectos secundarios. Las ciclodextrinas tienen la propiedad de complejar moléculas hidrófobas en su cavidad y solubilizarlas en medio acuoso, lo que ha sido extensamente utilizado en la industria farmacéutica. No obstante, existen bastantes limitaciones respecto al uso farmacológico de las ciclodextrinas naturales, debido a la baja solubilidad en agua de sus complejos de inclusión, su carácter hemolítico, y la carencia de elementos de vectorización hacia receptores biológicos. Aunque se han desarrollado y comercializado derivados de β-ciclodextrina que mejoran significativamente las propiedades de solubilidad y toxicidad de las ciclodextrinas nativas, las limitaciones relacionadas con el transporte no selectivo aún no han sido superadas. La puesta a punto de métodos eficaces para la incorporación de biomarcadores selectivos sobre ciclodextrinas, que faciliten el transporte vectorizado de fármacos, es esencial para que su

uso permita reducir la dosis, evitar los efectos secundarios y aumentar la eficacia de los fármacos transportados.

Una de las líneas de investigación del grupo en el que se ha desarrollado esta Tesis está orientada al diseño de glicomiméticos con estructura de sp^2-iminoazúcares adecuados para su aplicación en las terapias denominadas de "acompañante químico" (chaperonas farmacológicas, PCs) para el tratamiento de enfermedades de almacenamiento lisosomal (Lysosomal Storage Diseases, LSDs), con especial atención a la enfermedad de Gaucher. Aunque estos sp^2-iminoazúcares han mostrado una actividad prometedora como chaperonas, un factor limitante en su aplicación es su baja solubilidad en medio acuoso y, por tanto, la dificultad para alcanzar la concentración necesaria para obtener un efecto médicamente relevante. El uso de sp^2-iminoazúcares como fármacos viables requiere, en muchos casos, solucionar aspectos relativos a la solubilidad y estabilidad de los glicomiméticos en el medio biológico y a la posibilidad de conducirlos selectivamente a células o tejidos específicos. Concretamente, en el caso de la enfermedad de Gaucher las células más afectadas, y que constituirían la diana primaria, son los macrófagos.

En esta Tesis Doctoral, se ha sintetizado un derivado monofuncionalizado de la β-ciclodextrina al que se ha acoplado una antena dendrítica trimanosilada en una de sus posiciones primarias como elemento de biorreconocimiento ($(ManS)_3$-βCD). Este diseño, facilita la complejación de derivados de sp^2-iminoazúcares con cadenas lipófilas que contienen un segmento de *n*-octilo (6S-NOI-NJ) o de adamantilo (6S-NAdB-NJ), ya que deja libre el acceso a la cavidad hidrófoba de la ciclodextrina por la cara secundaria, más ancha, de estos fragmentos que de por sí presentan una elevada afinidad por la misma. Además, el dendrón manosilado trivalente es reconocido también de manera eficaz por el receptor específico de manosa presente en las membranas celulares de macrófagos (macrophage mannose receptor, MMR). La capacidad de complejación de los conjugados anfifílicos de sp^2-iminoazúcares por el transportador $(ManS)_3$-βCD se demostró mediante estudios de valoración por ^{1}H RMN en agua que demuestran que es efectivamente el

sustituyente lipófilo aglicónico el que se incluye en la ciclodextrina. Los ensayos de inhibición enzimática de los *sp*^{2}-iminoazúcares 6S-NOI-NJ y 6S-NAdB-NJ libres y formando complejos de inclusión con el transportador $(ManS)_3$-βCD frente a enzimas comerciales, como la β-glucosidasa de almendra y la β-galactosidasa de hígado de buey, indican que la actividad inhibitoria no se ve disminuida por la complejación. La capacidad de reconocimiento de la antena trimanosilada, tanto en el transportador $(ManS)_3$-βCD como en los correspondientes complejos, por lectinas específicas de manosa se ha evaluado primero mediante ensayos ELLA (*Enzyme-Linked Lectin Assay*) y valoración por calorimétrica isotérmica (ITC) utilizando la lectina concanavalina A (*Concanavalis Ensiformis*, Con A) como modelo. Se observó que el aumento de afinidad respecto al metil α-D-manopiranosido, utilizado como control, debido al efecto multivalente se conserva en los complejos. Estos resultados se han visto confirmados en los ensayos ELLA realizados con el receptor recombinante de manosa de macrófago humano (rhMMR).

La actividad chaperona de los *sp*^{2}-iminoazúcares 6S-NOI-NJ y 6S-NAdB-NJ y de sus complejos con $(ManS)_3$-βCD, evaluada frente a tres fibroblastos mutantes de pacientes de Gaucher (GD) y frente a fibroblastos nativos sanos, indica que su comportamiento es independiente de la complejación, lo que confirma que existe una transferencia rápida de la chaperona desde la cavidad de la βCD al sitio activo de la β-glucocerebrosidasa humana en los fibroblastos.

Para confirmar el potencial del conjugado $(ManS)_3$-βCD para el transporte dirigido, se ha llevado a cabo un estudio fluorimétrico *in vitro* de la capacidad de adhesión del transportador y sus complejos con las chaperonas a macrófagos peritoneales de ratón. Los resultados apoyan que el transportador y los correspondientes complejos compiten por el mismo receptor de la superficie de los macrófagos, de acuerdo con la participación del MMR en el proceso de adhesión. Con objeto de confirmar además si los complejos se

internalizan en macrófagos a través de receptores MMR, hemos diseñado un ensayo competitivo en macrófagos diferenciados a partir de monocitos humanos THP-1, usando microscopia de fluorescencia 3D. Este tipo de células presentan muchas propiedades análogas a las de los macrófagos humanos primarios, incluyendo la expresión del MMR. El conjunto de resultados apoya la hipótesis de que el derivado $(ManS)_3$-βCD interacciona específicamente con el receptor MMR a través de la antena trimanosilada y que esta interacción desencadena la internalización del correspondiente complejo de inclusión con la chaperona en el interior de los macrófagos.

GLICODERIVADOS CATIÓNICOS ANFIFÍLICOS CON CAPACIDAD DE AUTOORGANIZACIÓN COMO TRANSPORTADORES DE MATERIAL GÉNICO

La terapia génica es un procedimiento terapéutico prometedor para el tratamiento de una amplia gama de enfermedades, tanto de origen genético como adquirido, que consiste en introducir material génico (ADN o ARN) en células somáticas humanas para expresar o suprimir la expresión de una proteína determinada. Este proceso es conocido como transfección. La utilización directa del ADN o ARN libre para la transfección presenta importantes limitaciones, tales como la baja permeabilidad de la membrana plasmática y la inestabilidad metabólica del material génico, por lo que resulta imprescindible el diseño de sistemas transportadores de genes, conocidos como vectores de transfección, capaces de compactar, proteger y transportar el material génico hasta el núcleo celular. Aunque los sistemas de transfección basados en vectores virales han mostrado una alta eficiencia, su aplicación está muy limitada debido a los problemas de bioseguridad asociados a su manejo. En consecuencia, el diseño de vectores no virales ha experimentado un fuerte desarrollo en los últimos años. La mayoría de los vectores no virales recogidos en la literatura se basan en sistemas poliméricos catiónicos en los que su

composición intrínsecamente polidispersa constituye una importante desventaja para los estudios de estructura-actividad (SAR) y para su aplicación farmacológica. Como alternativa, en esta Tesis se han desarrollado sistemas moleculares bien definidos, con estructura de lípidos catiónicos basados en carbohidratos, capaces de autoorganizarse para complejar, condensar y proteger el material génico y liberarlo en las células. Se han desarrollado métodos de funcionalización selectiva para preparar vectores sintéticos anfifílicos, que tienen grupos catiónicos capaces de interaccionar electrostáticamente con los grupos fosfato de los nucleótidos y cadenas grasas hidrofóbicas que les proporcionan propiedades de autoensamblado para la condensación y liberación del material génico. Las plataformas en las que se han incorporado estos grupos funcionales han sido azúcares sencillos y comerciales como el metil α-D-glucopiranosido y la α,α'-trehalosa. Los grupos cargados positivamente y las cadenas hidrófobas han sido incorporadas tanto en las posiciones primarias de los azúcares como en las secundarias obteniéndose así vectores con los grupos catiónicos en las posiciones primarias y cadenas grasas en las posiciones secundarias (vectores tipo "falda"), o vectores denominados "medusa" en los que las cargas positivas se sitúan en las posiciones secundarias de los azúcares, lo que les proporciona una mayor carga hidrófila. Se ha obtenido una amplia colección de vectores sintéticos en los que se ha modificado el grupo funcional de anclaje de las cargas positivas (triazol/tiourea), el número y disposición de éstas (lineal o dendrítica); así como la longitud y naturaleza de las cadenas hidrófobas (alquílicas/acílicas). Estos vectores sintéticos han mostrado capacidad para autoorganizarse en presencia de ADN y promover su compactación en experimentos de electroforesis en gel de agarosa y dispersión dinámica de la luz (DLS), observándose que los derivados de trehalosa con presencia de grupos tiourea son los más eficaces para este fin.

En el marco de un proyecto más amplio, la capacidad de transfección de los compuestos preparados en esta Tesis se ha evaluado en comparación con derivados diméricos cíclicos de trehalosa, denominados ciclotrehalanas (CTs), en los que una de las

unidades disacarídica soporta las cadenas grasas y la otra los grupos amino protonables. De manera análoga a las ciclodextrinas policatiónicas anfifílicas (paCDs), que han mostrado una gran capacidad de transfección génica, las ciclotrehalanas policatiónicas anfifílicas (paCTs) están dotadas de anfifilicidad facial, con dos dominios claramente diferenciados separados por la estructura macrocíclica. Los experimentos de DLS y electroforesis en gel de agarosa mostraron que estos derivados forman pequeñas particulas de tamaño nanométrico y menor polidispersidad que las de los derivados anfifílicos de metil α-D-glucopiranosido y α,α'-trehalosa y, además, con mejores propiedades de compactación y protección del ADN. Cuando se compara la eficiencia de transfección de los derivados anfifílicos de metil α-D-glucopiranosa, α,α'-trehalosa y paCTs en las líneas celulares COS-7 (células de riñón de primate) y HepG2 (hepatoblastoma humano), utilizando el gen reportero de luciferasas eFLuc, se concluye que las paCTs son significativamente menos tóxicas que los derivados anfifílicos de metil α-D-glucopiranosa y α,α'-trehalosa y que se necesitan dosis menores de compuesto para compactar los plásmidos. Además, las paCTs son mucho más eficaces en la transfección para cualquiera de las dos líneas celulares, manteniendo la eficacia incluso en presencia de concentraciones significativas de suero bovino fetal (FBS). Las excelentes propiedades como transportadores de genes de las paCTs, comparables a la de las ciclodextrinas policatiónicas anfifílicas, hacen de estos derivados buenos candidatos para su aplicación en ensayos *in vivo* en terapias contra el cáncer.

DERIVADOS ANFIFÍLICOS DE TREHALOSA Y GLUCOSA COMO MODULADORES DE LA ACTIVIDAD DEL SISTEMA INMUNE INNATO

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and Nanoparticle Toll-Like Receptor 4 (TLR4) Modulators, *J. Med. Chem.* **2014**, *57*, 9105-9123.

La modulación (activación/inhibición) del receptor TLR4 (Toll-like receptor 4) juega un papel muy importante en los mecanismos de respuesta inmune innata. La activación de los receptores TLR4 está asociada a ciertas enfermedades autoinmunes, a desórdenes inflamatorios no infecciosos y a daños neuronopáticos, lo que implica un amplio rango de aplicaciones clínicas posibles para los antagonistas sintéticos de este receptor. La mayoría de estos moduladores del TLR4 son moléculas pequeñas relacionadas con el lípido A, un lipodisacárido fosforilado cargado negativamente que es el responsable de la activación del TLR4 a través de procesos de reconocimiento específico. En general, se trata de lípidos aniónicos que portan uno o dos grupos fosfatos o grupos isostéricos de los fosfatos (carboxilatos o sulfatos) con un dominio hidrofóbico constituido por cadenas lipídicas (cadenas alquílicas o esteroides) y en los que el núcleo disacarídico típico del lípido A puede ser sustituido por otro soporte sacarídico o no sacarídico. Sorprendentemente, ciertos glicolípidos catiónicos han demostrado también un papel activo en la modulación de la actividad del TLR4, aunque los mecanismos moleculares que rigen esta actividad son todavía desconocidos debido al escaso número de ejemplos estudiados hasta la fecha. En esta Tesis, hemos utilizado las metodologías de funcionalización selectivas de carbohidratos puestas a punto para diseñar una serie amplia de glicoderivados anfifílicos catiónicos y aniónicos tipo "falda" utilizando el metil α-D-glucopiranosido y la α,α'-trehalosa como plataformas. Estudios comparativos de estructura-actividad (SAR) utilizando esta colección de moléculas anfifílicas nos ha permitido establecer qué elementos estructurales son esenciales para acceder a moduladores sintéticos eficaces de la actividad del TLR4. En primer lugar, se han llevado a cabo estudios de la capacidad de autoorganización en agua utilizando valoraciones fluorimétricas para determinar las concentraciones micelares críticas (CMCs) y experimentos de DLS para determinar el tamaño y carga superficial de las nanopartículas.

Estos datos son interesantes para determinar si el papel modulador de la actividad de TLR4 es ejercido por moléculas individuales o por agregados supramoleculares. Los estudios de modulación de la actividad del TLR4 en células embrionarias de riñón humanas (HEK-Blue™) demuestra que los compuestos con cadenas hidrofóbicas acílicas son los únicos que inhiben la actividad del TLR4. Entre estos, son los derivados de trehalosa catiónicos los que muestran una mayor potencia de inhibición. Estos derivados anfifílicos de trehalosa han demostrado su capacidad de inhibir eficazmente la activación del TLR4 en células HEK-293 transfectadas con genes MD2-TLR4 de roedor y humano. Algunos de los candidatos seleccionados muestran también esta actividad inhibidora en macrófagos de médula de roedor (BMDM). En ensayos *in vivo* con ratones, todos los derivados de trehalosa anfifílicos seleccionados inhiben la activación de su sistema inmune por el LPS.

Además, se ha puesto a punto un procedimiento para soportar los mejores candidatos como inhibidores de la actividad TLR4 sobre nanoparticulas de oro (AuDDT), consiguiendo así una presentación multivalente. Aunque los ensayos de inhibición de la actividad del TLR4 en células HEK-BlueTM muestran que las nanopartículas preparadas a partir de glicolípidos catiónicos conservan su actividad, su alta toxicidad celular previene de su uso para ensayos *in vivo* con ratones. En consecuencia, parece más conveniente el uso directo de los glicolípidos catiónicos como moduladores de la activación del sistema inmune en ensayos *in vivo*. En el caso de los glicolípidos aniónicos los experimentos de valoración fluorimétrica y de DLS indican que poseen propiedades de autoorganización similares a sus análogos catiónicos. Se espera que los ensayos de modulación de la activación del TLR4 que se están realizando en colaboración con el Prof. Peri nos permitan determinar si actúan como inhibidores o activadores de los mecanismos de respuesta inmune.

Summary.

In this Thesis we have synthesized β-cyclodextrin, methyl α-D-glucopyranose and α,α'-trehalose derivatives tailored for use in targeting drug delivery, gene transfection and/or innate immune system modulation. For targeting drug delivery a convergent synthesis strategy has been applied to obtain a monofunctionalized β-cyclodextrin precursor as complexing platform for low water soluble drugs in which a biorecognition antenna is inserted to ease targeted drug transport; in this case pharmacological chaperones with sp^2 iminosugar structures.

To prepare gene delivery vectors, methyl α-D-glucopyranose and α,α'-trehalose has been used as saccharidic platforms in which structural elements to promote self-assembling has been incorporated by selective functionalization methodologies. Thus, cationic mono- and disaccharides with amphiphilic character enable to form nanoparticle either spontaneously or in plasmid presence. This versatile synthetic approach allows us to obtain a molecule collection with high structural variability which has been subjected to studies to establish relations between their molecular structure and their self-assembling and gene delivery properties.

Cationic α-D-glucopyranose and α,α'-trehalose derivatives, synthesized by selective functionalization methods, show structural resemblance with charged amphiphilic molecules that play an important role in modulating innate immune system by interaction with the toll-like receptor TLR4. Since both cationic and anionic derivatives are susceptible to act as TLR4 activity regulators, we have also optimized the synthesis of amphiphilic anionic analogues and, in collaboration with Prof. F. Peri (Universidad de Milano Biccoca), we have assessed the modulation activity of immune response on both glycolipid families.

CYCLODEXTRINS WITH BIORECONOCIBLE ANTENAE FOR TARGETING TRANSPORT OF PHARMACOLOGICAL CHAPERONES

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The design of systems for condensation and controlled release of active principles is essential to implement novel therapies oriented to improve the bioavailability of insoluble or biodegradable medicaments and to avoid side effects. Cyclodextrins are capable to complex and solubilize hidrophobic molecules inside their cavities in aqueous media what has extensively used in pharmaceutical industry. However, natural cyclodextrins have several drawbacks for their pharmacological uses due to the low solubility of their inclusion complexes, their hemolytic character, and the lack of targeting domains towards biological receptors. Development of methods to insert selective biomarkers on cyclodextrins that facilitate targeted drug delivery is essential for lowering drug dose, avoiding side effects and enhancing efficiency of the transported drugs.

One of the main research lines in our group is focused on the design of glycomimetics with sp^2-iminosugar structure suitable for implementation of the so-called "chemical chaperone therapies" (pharmacological chaperones, PCs) to treat lysosomal storage diseases (LSDs), such as Gaucher disease, Fabry disease, etc. Although the sp^2-iminosugars have shown a promising activity as chaperones, a limiting factor for their application is their low water solubility and, as a consequence, the difficulty to reach the required concentration to obtain a medically relevant effect. The use of sp^2-iminosugar as feasible drugs needs, in many cases, to overcome issues related to the solubility and

stability of glycomimetics in aqueous biological environment as well as the possibility of selectively leading them to specific cells and tissues. Particularly, in the case of Gaucher disease (GD), leading them to the cells most affected, the macrophages, would be the prior aim.

In this PhD Thesis a monofunctionalized β-cyclodextrin derivative in which a trimannosylated dendritic antenna at its primary positions has been inserted as biorecognition domain ($(ManS)_3$-βCD). This design facilitates the complexation of the sp^2-iminosugar derivatives with lipophilic chains containing *n*-octyl (6S-NOI-NJ) or adamantyl (6S-NAdB-NJ) segments because it leaves open access to the cyclodextrin wider hydrophobic cavity to these fragments that present a high affinity for this cavity. Moreover, the trivalent mannosylated dendron is efficiently recognized by the macrophage mannose receptors (MMR). The complexing capacity of the amphiphilic sp^2-iminosugar conjugates towards $(ManS)_3$-βCD was proved by ^{1}H NMR titration studies that confirm the lipophilic aglyconic substituent is included in the cyclodextrin. The enzymatic inhibition assays of the free sp^2-iminosugars 6S-NOI-NJ and 6S-NAdB-NJ and the corresponding inclusion complexes with the $(ManS)_3$-βCD carrier against commercial enzymes such as almond β-glucosidase and bovine liver β-galactosidase indicate that inhibitory activities do not decrease because of complexation. The recognition capability of the trimannosylated antenna either in $(ManS)_3$-βCD carrier or in the corresponding complexes by mannose specific lectins was firstly assessed by enzyme-linked lectin assays (ELLAs) and isothermal titration calorimetry (ITC) using Concanavalin A lectin (*Concanavalis Ensiformis*, Con A) as model receptor. Conservation of affinity enhancement of mannosylated dendron domain in comparison with methyl α-D-mannopyranoside in the complexes was observed. These results have been confirmed by the ELLA assays carried out with the recombinant human mannose macrophage receptor (rhMMR).

The chaperone activity of the sp^2-iminosugars 6S-NOI-NJ and 6S-NAdB-NJ and their corresponding complexes with $(ManS)_3$-βCD has been assessed against three mutant fibroblasts of GD patients indicating that their behavior is independent of the complexation, what confirms the existence of a fast chaperone transfer from βCD cavity to β-glucocerebrosidase active site in the fibroblasts.

To confirm the potential of the $(ManS)_3$-βCD conjugate for targeting delivery, a fluorimetric study *in vivo* about adhesion capacity of the carrier and their complexes with chaperones against peritoneal murine macrophages was achieved. Results support that the carrier and the corresponding complexes compete by the receptor on the macrophage surface in agreement with MMR participation in the adhesion process. In order to further confirm whether the complexes are internalized inside macrophages through MMR receptors we have designed a competitive assay in differentiated macrophages from human monocytes THP-2 using 3D fluoresce microscopy. This type of cells has many similar properties to prior human macrophages, including MMR expression. The set of results supports the hypothesis that $(ManS)_3$-βCD derivative specifically interact with MMR receptor through trimannosylated antenna and this interaction triggers internalization of the corresponding inclusion complex with the chaperone inside of macrophages.

CATIONIC AMPHIPHILIC GLYCODERIVATIVES WITH SELF-ASSEMBLY AS GENE DELIVERIES

The gene therapy is a promising therapeutic procedure to treat a large range of diseases, both genetic and acquired ones, which comprises introducing gene material (DNA or RNA) in somatic human cells to express or suppress the expression of a specific protein. This process is called transfection. Direct use of naked DNA or RNA for transfection has important limitations, such as the low permeability of the plasmatic

membrane and metabolic instability of the gene material, therefore it is essential the design of gene delivery systems, so-called transfection vectors, capable of compacting, protecting and transporting gene material to cell nucleus. Although transfection systems based on viral vectors have shown high transfection efficiency, their application is rather limited due to biosafety issues associated with handling. Most of non-viral vectors collected in bibliography are based on cationic polymeric systems in which their inherently polydisperse composition represents a major drawback for studies of structure-activity relationship (SAR) and for pharmacological application. Alternatively, in this Thesis well-defined molecular systems with cationic lipid structure based on carbohydrates, capable of self-assembling to complex, condensate and protect gene material, and to release it in cells, have been developed. Selective functionalization methodologies have been optimized to prepare synthetic amphiphilic vectors containing cationic groups that are able to interact electrostatically with the nucleotide phosphate groups, and hydrophobic fatty chains which give them self-assembly capabilities to condensate and release the gene material. The selected scaffolds are simple and commercial sugars such as methyl α-D-glucopyranoside and α,α'-trehalose. Positively charged groups and hydrophobic chains have been inserted both in primary and secondary positions of the sugars to obtain vectors with cationic groups at secondary positions and fatty chains at secondary positions (vectors type "skirt") or vectors so-called "jelly-fish" in which positive charges are situated on the sugar secondary positions what provides them with higher hydrophilic character. A wide collection of synthetic vectors in which the anchoring functional group (triazole/thiourea) of positive charges, the number and disposition have been varied as well as the length and nature of the hydrophobic chains (alkyl/acyl) have been prepared. These synthetic vectors have proved ability to self-assembly in the presence of DNA and promote their compaction in agarose gel electrophoresis and dynamic light scattering (DLS) experiments. The ensemble of data may conclude that trehalose derivatives functionalized with thiourea groups are the most effective for this purpose.

Within the framework of a larger project, the transfection ability of the compounds prepared in this Thesis have been assessed in comparison with cyclic dimeric trehalose derivative, so-called cyclotrehalans (CTs), in which one of the disaccharidic residues incorporates fatty chains, and the other, protonable amine groups. Similarly to policationic amphiphilic cyclodextrins (paCDs), that have shown large gene delivery capacity, the polycationic amphiphilic cyclotrehalans (paCTs) are endowed with facial amphilicity, with two clearly distinct domains separated by the macrocyclic structure. The DLS and agarose gel electrophoresis experiments showed these derivatives form nanometric size nanoparticles with lower polydispersities than the amphiphilic derivatives of methyl α-D-glucopyranoside and α,α'-trehalose, and with better compaction and protection properties of DNA. Comparison of transfection efficiencies between amphiphilic methyl α-D-glucopyranoside and α,α'-trehalose derivatives and paCTs in COS-7 cells (monkey kidney cells) and HepG2 cells (human hepatoblastoma cells) using luciferase eFLuc as reporter gene demonstrate the paCTs are significantly less toxic than amphiphilic methyl α-D-glucopyranoside and α,α'-trehalose derivatives and lower dose of paCTs compounds are needed to compact the plasmids. Moreover the paCTs are more efficient for transfection in both cell lines, maintaining the efficiency even in the presence of relevant concentrations of bovine fetal serum (FBS). The excellent features of paCTs as gene carriers, comparable to those of paCDs, make these derivatives be good candidates for applying *in vivo* trials for cancer therapies.

TREHALOSE AND GLUCOSE AMPHIPHILIC DERIVATIVES AS MODULATORS OF INNATE IMMUNE SYSTEM ACTIVITY

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and Nanoparticle Toll-Like Receptor 4 (TLR4) Modulators, *J. Med. Chem.* **2014**, *57*, 9105-9123.

Modulation (activation/inhibition) of TLR4 receptor (Toll-like receptor 4) plays an important role on innate immune response mechanisms. The activation of TLR4 receptors is related to certain self-immune diseases, non-infectious inflammatory disorders and neuropathic damages, which implies a broad range of possible clinic applications for synthetic antagonists of this receptor. Most TLR4 modulators are small molecules structurally related to lipid A, a negatively charged phosphorylated liposaccharide that is responsible of TLR4 activation through specific recognition processes. In general, synthetic modulators are anionic lipids bearing one or two phosphate groups or phosphate isosteric groups (carboxylates or sulphates) with a hydrophobic domain comprising lipidic chains (alkyl chains and steroids) and in which the typical disaccharidic nucleus of lipid A can be replaced by other saccharidic or non-saccharidic scaffolds. Surprisingly, certain cationic glycolipids have also shown an active role in TLR4 activity modulation, although the molecular mechanisms that govern this activity are still unknown due to the scarce number of examples studied so far. In this Thesis, we have used the previously optimized selective functionalization methodologies of carbohydrates to design a broad range of cationic and anionic amphiphilic "skirt"-type glycoderivatives with α-D-glucopyranoside and α,α'-trehalose as saccharide platforms. Comparative structure-activity relationship (SAR) studies using this collection of amphiphilic molecules have allowed us to determine the essential structural elements to construct efficient synthetic modulators of TLR4 activity. Firstly, self-assembling ability in water studies have been carried out using fluorimetric titrations to determine critic micellar concentratios (CMCs) and DLS experiments to estimate size and charge surface of the nanoparticles. These data are interesting to elucidate if modulation of TLR4 activity is exerted by individual molecules or by supramolecular aggregates. The studies of modulating activity of TLR4 in human embryonic kidney cells (HEK-Blue™) demonstrate that compounds bearing

hydrophobic acyl chains are the only ones that inhibit the activity of TLR4. These amphiphilic trehalose derivatives have demonstrated their ability to inhibit efficiently the activity of TLR4 in HEK-293 cells transfected with murine and human MD2-TLR4 genes. Several selected candidates also show this inhibitory activity in murine bone-marrow derived macrophage cells (BMDM). In *in vivo* assays with mice, all amphiphilic trehalose derivatives selected inhibit the immune system activation by LPS.

Moreover, a procedure for supporting on gold nanoparticles (AuDDT) the best candidates as inhibitors of TLR4 activity has been set up to produce a multivalent arrangement. Although inhibition assays of TLR4 activity in HEK-Blue TM cells indicate that the nanoparticles keep their activity, their high cell toxicity could prevent their use in *in vivo* assays with mice..Consequently, it seems more convenient the direct use of cationic glycolipid molecules as modulators of activation of immune system in *in vivo* experiments. In the case of the anionic glycolipids, fluorimetric titration and DLS experiments indicate that these compounds have self-assembly properties similar to their cationic analogues. It is expected the assays of modulating activation of TLR4 that are currently running in collaboration with Prof. Peri allow us to establish if they act as inhibitors or activators of the immune response mechanisms.

Capítulo 1

Introducción general

1. Introducción general.

Las investigaciones encaminadas a la mejora de la biodisponibilidad de principios activos en medio fisiológico, incluyendo el diseño de derivados con propiedades óptimas para su utilización como fármacos y el transporte de agentes terapeúticos hacia los centros de acción deseados, han repercutido de manera decisiva en el progreso hacia nuevas terapias en áreas como la oncología,[1] las enfermedades poco frecuentes[2] o las enfermedades infecciosas.[3] Dada la gran importancia que tienen los fenómenos de reconocimiento de carbohidratos con otras biomoléculas, especialmente proteínas, en procesos biológicos y patológicos, los derivados sintéticos de carbohidratos (glicomiméticos) muestran un gran potencial para estos fines. Las posibilidades de aplicación de los estudios de interacciones celulares mediadas por glicoconjugados y el desarrollo de glicomiméticos que permitan su control abarcan campos como la defensa inmunológica, la replicación vírica, la inflamación, la transducción de señales bioquímicas o la adhesión de agentes microbianos a una célula huésped, entre otros (Figura 1.1.)[4,5]

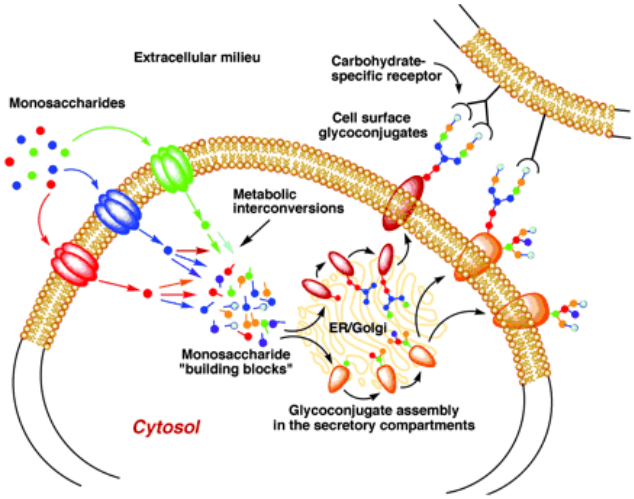


Figura 1.1. Biosíntesis y reconocimiento en la superficie celular de glicoconjugados.[4]

En este contexto, la Tesis que se presenta aborda nuevas estrategias para la preparación de glicomiméticos específicamente diseñados para desempeñar funciones que redunden en la mejora de la calidad de vida, ya sea regulando la actividad de enzimas implicadas en procesos patológicos (*glicofármacos*) o permitiendo el transporte de principios activos en el organismo (*glicotransportadores*).

1.1 Glicofármacos

Los oligosacáridos y glicanos (glicoproteínas, glicolípidos, proteoglicanos) son constituyentes fundamentales de los organismos vivos, jugando un papel esencial en procesos como el plegamiento, tráfico y control de calidad de proteínas, la diferenciación y proliferación celular o la comunicación intercelular, incluyendo el reconocimiento por patógenos, o la respuesta inmunológica.[4,5] Las estructuras de estos carbohidratos se remodelan por la acción de glicosiltransferasas y glicosil hidrolasas (glicosidasas), de una manera que es específica tanto respecto a la proteína como al tejido en cuestión. El funcionamiento correcto de estas enzimas refleja el estado fisiológico de la célula y la disfunción de alguna de ellas se traduce, en la mayoría de los casos, en la existencia de una patología. Consecuentemente, los de compuestos capaces de interferir con las enzimas que procesan carbohidratos presentan un alto potencial como fármacos.

En los últimos veinte años se han realizado avances decisivos en la comprensión del papel de los carbohidratos, sus receptores y los procesos en que intervienen en el funcionamiento de los sistemas biológicos. La diversidad estructural de los carbohidratos proporciona una gran oportunidad para la identificación de nuevas dianas terapéuticas y el desarrollo de nuevas terapias. Sin embargo, los carbohidratos raramente se pueden emplear para este propósito debido a su inestabilidad metabólica y a su rápida degradación in vivo. En consecuencia, las investigaciones se han dirigido a identificar análogos de hidratos de carbono, glicomiméticos, tanto procedentes de fuentes naturales

como sintéticos,[6,7,8] con mayor estabilidad, afinidad y eficacia para el desarrollo de aplicaciones clínicas y su utilización como *glicofármacos*. Entre estos, los iminoazúcares (iminociclitoles), en los que formalmente el oxígeno endocíclico característico de los monosacáridos ha sido sustituido por un átomo de nitrógeno, son probablemente los glicomiméticos más estudiados hasta la fecha, siendo ampliamente utilizados en glicobiología tanto en investigación básica como aplicada (Figura 1.2.).[6,9,10]

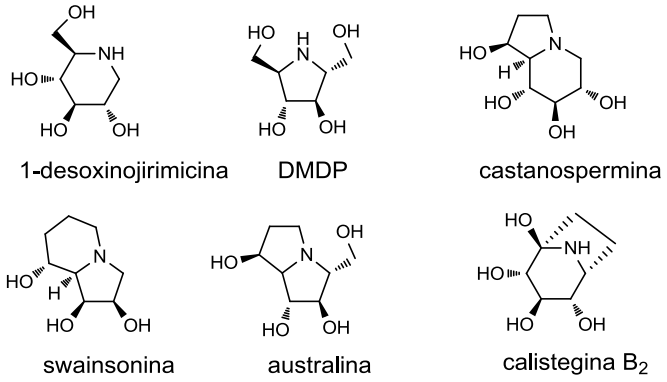


Figura 1.2. Estructuras de algunos de los alcaloides polihidroxilados más representativos de las diferentes familias de iminoazúcares naturales.

A pesar de que muchos iminoazúcares han mostrado propiedades interesantes para su posible aplicación en el tratamiento de diferentes patologías, su traslación a la clínica se ha visto limitada en muchos casos por falta de selectividad o de estabilidad. Con objeto de superar estos problemas, el grupo de investigación en el que se ha realizado esta Tesis desarrolló el concepto de ‘’*sp*^{2}-iminoazúcar’’. En los *sp*^{2}-iminoazúcares, el nitrógeno imínico *sp*^{3} de los iminoazúcares clásicos es sustituido por un átomo de nitrógeno de tipo pseudoamida (tiourea, urea, isourea, isotiourea, carbamato, tiocarbamato o guanidina) con un importante carácter *sp*^{2}. Este cambio estructural aumenta la contribución orbitálica al efecto anomérico en el centro aminoacetálico, permitiendo acceder a *sp*^{2}-iminoazúcares estables, reductores o no reductores, que muestran integridad conformacional y

configuracional en agua. Además, esta estrategia facilita la incorporación de sustituyentes exocíclicos con orientaciones bien definidas, favoreciendo así interacciones no glicónicas con aminoácidos localizados en la vecindad del sitio catalítico de enzimas complementarias. De esta manea se han diseñado inhibidores de glicosidasas que muestran una elevada selectividad de acción y mejores propiedades como glicofármacos (Figura 1.3.).[11,12,13,14,15]

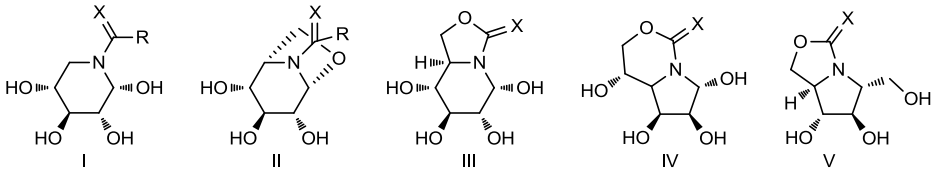


Figura 1.3. Estructura de algunos tipos de sp^2-iminoazúcares (X = O, S, NHR).

Aunque los efectos biológicos de los iminoazúcares están asociados en gran medida a su actividad como inhibidores de glicosidasas, en algunos casos la actividad biológica no puede explicarse exclusivamente en base a ésta. Por ejemplo, las dianas tradicionales para el uso de iminoazúcares en oncología son enzimas implicadas en el procesado de glicoconjugados de la superficie celular, tales como glucosidasas, manosidasas o fucosidasas. Sin embargo, los diferentes efectos anticancerosos observados para varios iminoazúcares, tales como la supresión del crecimiento celular, la actividad antimetastática, la regulación inmunológica, la actividad antiangiogénica o la inducción de apoptosis, sugieren la existencia de diferentes mecanismos de acción que pueden o no estar directamente relacionados con la inhibición de glucosidasas.[8] Recientemente se han identificado un número creciente de dianas terapéuticas relacionadas con el reconocimiento o procesado de carbohidratos y susceptibles de interaccionar con glicomiméticos, tales como proteínas solubles, enzimas que procesan inositoles o sitios polares activos (Figura 1.4.). Además la elucidación de nuevos procesos promovidos por iminoazúcares, como la asistencia al plegamiento correcto de proteínas que reconocen carbohidratos, se ha traducido en una expansión constante del potencial médico de estos

glicomiméticos. En el contexto de esta Tesis nos centraremos en dos aplicaciones biomédicas concretas, a saber, las enfermedades de depósito lisosomal y el desarrollo de moduladores del sistema inmune innato y los mecanismos de inflamación.

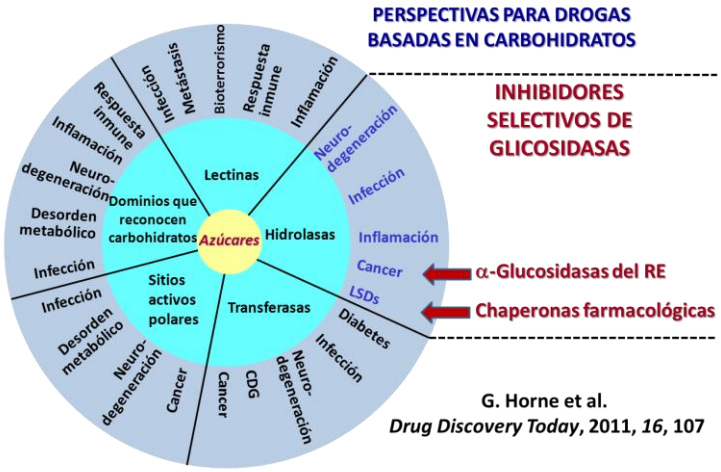


Figura 1.4. Perspectivas para el desarrollo de fármacos basados en carbohidratos y glicomiméticos.

Bajo el término ‘’enfermedades de almacenamiento lisosomal’’ (**Lysosomal Storage Diseases, LSDs**) existen descritos más de 50 desórdenes genéticos caracterizados por la disfunción de proteínas lisosomales implicadas en la degradación metabólica de determinados glicoconjugados.[16,17] Se estima que afectan a 1 de cada 6000 nacimientos en los países europeos[18] y están causadas por la pérdida total o parcial de actividad de hidrolasas o por alteraciones en las proteínas implicadas en las modificaciones post-traslacionales de las enzimas lisosomales. Los síntomas que derivan de la acumulación intralisosomal de macromoléculas no degradadas causan daños a los órganos afectados y conducen a menudo a la muerte. Recientemente se ha desarrollado una nueva terapia que consiste en la administración oral de un compuesto, denominado chaperona farmacológica (*Pharmacological Chaperone*, PC), capaz de incrementar la actividad residual de la enzima mutante en el lisosoma.[19,20,21,22] Muchas de las mutaciones en la

secuencia de aminoácidos de la enzima lisosomal que están en el origen de una LSD provocan que ésta no se pliegue correctamente y, si las chaperonas moleculares del retículo endoplasmático (RE) no son capaces de restaurar la conformación nativa de la enzima, ésta no supera el control de calidad del RE y es degradada por el proteosoma. Las PCs sirven de molde, favorecen el plegamiento correcto de la enzima mutante y ayudan al mantenimiento de la conformación nativa de la misma, facilitando el tráfico hasta los lisosomas (Figura 1.5).

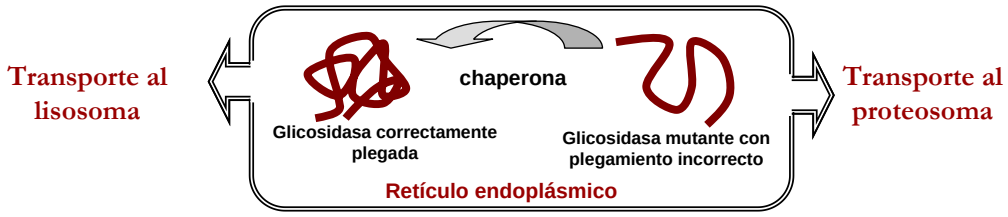


Figura 1.5. Representación esquemática de la estrategia de PCT para el tratamiento de LSDs.

En este sentido, los glicomiméticos con estructura de sp^2-iminoazucar han demostrado su idoneidad para su aplicación como PCs para el tratamiento de LSDs tales como la enfermedad de Gaucher.[23,24] Sin embargo, un factor limitante asociado a estos tratamientos es la baja biodisponibilidad de los agentes terapéuticos y, por tanto, la dificultad para alcanzar la concentración necesaria para su actividad.[25] El desarrollo de glicofármacos viables, incluso para estructuras que muestren grados de actividad elevados en evaluaciones *in-vitro* y *ex-vivo*, requeriría solucionar aspectos relativos a la solubilidad y estabilidad de los glicomiméticos en el medio biológico y a la posibilidad de conducirlos selectivamente a células o tejidos específicos.

Los receptores TLR (Toll-like receptors) son proteínas de transmembrana tipo I que juegan un papel crucial en el reconocimiento de patrones moleculares asociados a patógenos tales como virus, bacterias, protozoos y hongos, y en la posterior iniciación de la respuesta del sistema inmune innato.[26] Entre los TLRs, el TLR4 ha emergido como una importante diana terapéutica relacionada con un amplio número de enfermedades actuales

tales como asma, diabetes, obesidad, Alzheimer, esclerosis lateral amilotrófica, dermatitis, psoriasis, algunos tumores, y desórdenes cardiovasculares o problemas neuroinflamatorios y autoinmunes.[27] Como la mayoría de estas patologías carecen de un tratamiento farmacológico específico, moléculas sencillas capaces de modular la activación del TLR4 están atrayendo un creciente interés para su aplicación en los tratamientos clínicos de estas enfermedades. La mayoría de estos moduladores del TLR4 son moléculas pequeñas relacionadas con el lípido A (Figura 1.6.),[28,29] un lipodisacárido fosforilado cargado negativamente que es el responsable de la activación del TLR4 a través de procesos de reconocimiento específico.

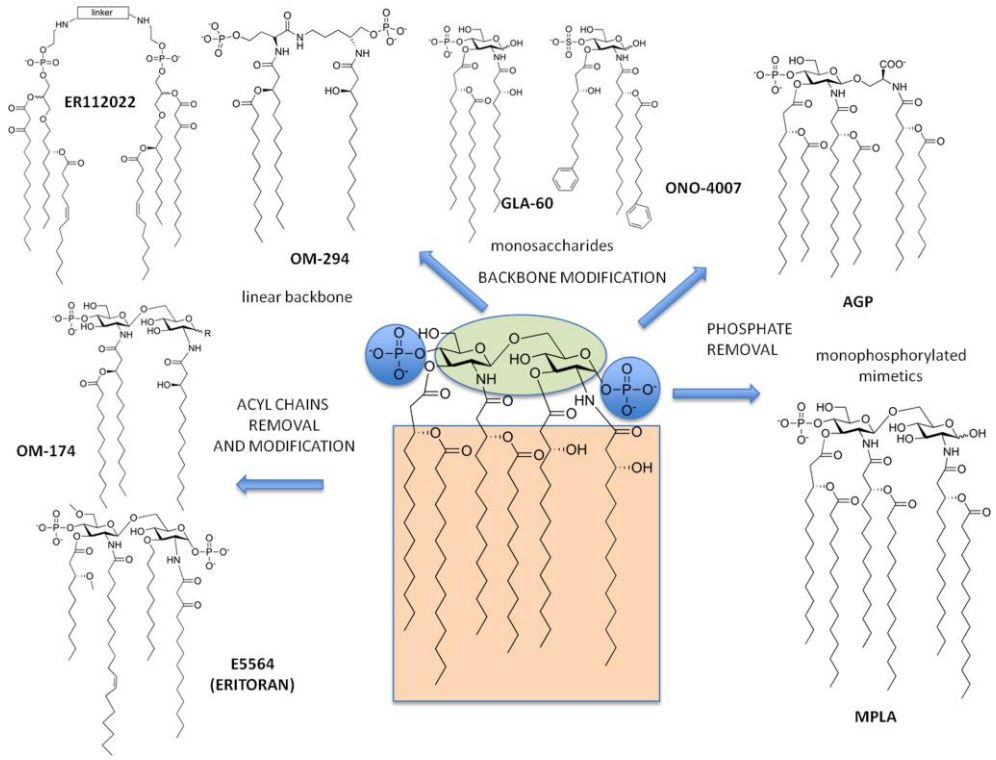


Figura 1.6. Moduladores síntéticos del TLR4 relacionados con la estructura del Lípido A procedente de la bacteria *E. coli*.[30]

En general, los miméticos sintéticos del lípido A son lípidos aniónicos que portan una o dos cargas negativas en grupos fosfatos o isostéricos de los fosfatos (carboxilatos o sulfatos) con un dominio hidrofóbico constituido por cadenas lipídicas (cadenas alquílicas o esteroides) en que el disacárido β-(1→6)-diglucosamina puede ser sustituido por otro soporte sacarídico o no sacarídico.[28,29,30] Estos miméticos aniónicos pueden actuar como antagonistas del TLR4 en procesos proinflamatorios de respuesta inmune (eritoran, Figura 3.6.)[31] o como agonistas (ONO1004, Figura 3.6).[32] Sin embargo, no sólo los miméticos aniónicos han mostrado capacidad de modulación de los procesos de respuesta inmune en los que está implicado el TLR4 sino que moléculas anfifílicas cargadas positivamente han demostrado también su eficacia como moduladores de estos procesos.[33] En consecuencia, en el contexto de esta Tesis se ha abordado el diseño de una amplia colección de glicomiméticos anfifílicos catiónicos y aniónicos, como moduladores de la actividad del TLR4 en procesos de respuesta inmune para su aplicación como glicofármacos en enfermedades relacionadas con estos procesos.

1.2. Glicotransportadores

Para ser farmacológicamente activos, todos los medicamentos deben poseer un cierto grado de solubilidad en agua, y la mayoría de ellos deberían ser lipófilos para facilitar su permeación a través de la membrana celular *via* mecanismos pasivos de difusión. Los nuevos métodos para el desarrollo de fármacos han permitido acceder a numerosas moléculas lipófilas con altas posibilidades terapéuticas pero cuyos ensayos clínicos no son posibles por su insolubilidad en agua. Por este motivo, una amplia variedad de macromoléculas con capacidad de carga, tales como oligonucleótidos, péptidos, proteínas y polímeros, han sido investigados como transportadores de fármacos. Sin embargo, en muchos casos su aplicación presenta dificultades, sobre todo en cuanto a biocompatibilidad.[34] Una alternativa la constituyen los compuestos moleculares con estructura macrocíclica que disponen de una cavidad adecuada para el alojamiento de otra especie huésped, formando así complejos de inclusión. La mayoría de los transportadores

elaborados por los químicos supramoleculares para el transporte de fármacos están basados en los oligosacáridos cíclicos de la familia de las ciclodextrinas. Si bien por lo general estos derivados carecen de elementos de reconocimiento biológico, lo que limita su aplicación terapéutica, es posible dotarlos de antenas de biorreconocimiento que, en muchos casos, también son de tipo sacarídico.[35] La utilización de este tipo de glicoconjugados transportadores de fármacos (glicotransportadores) facilita en muchos casos su transporte a través de la membrana celular y, a la vez, permite mejorar su biodisponibilidad y direccionarlo a un determinado tejido.[35]

La posibilidad de funcionalización selectiva de las ciclodextrinas también puede explotarse para promover su autoasociación en presencia de una especie activa, ampliando de esta manera las posibilidades de encapsulación más allá de las que determinan el tamaño de su cavidad.[36] Una estrategia que ha demostrado ser especialmente eficiente para este fin consiste en dotar a la molécula con características de anfifilia facial, lo que se consigue instalando dominios hidrófilos e hidrófobos en las opuestas del macrociclo.[37,38] En principio, este estrategia sería extensible a otros motivos sacarídicos que presentasen una diferenciación facial pronunciada, no necesariamente macrocíclicos, ampliando así la batería de glicotransportadores accesibles. En esta Tesis hemos explorado las dos vías. Por una parte, nos hemos centrado en el diseño y evaluación biológica de glicotransportadores de chaperonas farmacológicas basados en la β-ciclodextrina (βCD) y, por otra, en el estudio de nuevos sistemas supramoleculares nanométricos basados en mono y disacáridos anfifílicos y su posible utilización como vectores de transfección de genes.

Los derivados de ciclodextrinas han mostrado un gran potencial en la industria farmacéutica.[39] Además, los glicoconjugados de CDs han desempeñado un papel fundamental en el estudio de diferentes tipos de interacciones implicadas en el reconocimiento de carbohidratos o en la identificación de motivos individuales de reconocimiento.[4,40] Las CDs nativas son ciclomaltooligosacáridos de origen natural

formados por unidades de glucosa unidas por enlaces glicosídicos α(1→4). Están presentes en la naturaleza como azúcares de reserva de ciertos microorganismos que las obtienen por degradación enzimática de almidón. Presentan una estructura troncocónica toroidal, con una cavidad relativamente hidrófoba de dimensiones nanométricas (Figura 3.7.). Esta característica estructural otorga a las ciclodextrinas la capacidad de formar complejos de inclusión con moléculas apolares de tamaño apropiado permitiendo la solubilización de estos complejos en disolución acuosa debido al carácter hidrófilo de la cara exterior del anillo. Esta propiedad de encapsulamiento es utilizada en numerosos procesos industriales de interés farmacéutico, tecnológico y analítico.[41] Las principales ventajas de las CDs en este sentido son:

- Presentan una estructura bien definida.
- Permiten modificaciones químicas selectivas de su superficie externa.
- Existen diferentes tamaños de cavidad disponibles.
- Poseen bajos índices de toxicidad y actividad farmacológica.
- Mejoran la solubilidad de la molécula huésped en medio fisiológico.
- Pueden aumentar la biodisponibilidad y estabilidad del fármaco.

Las CDs comercialmente accesibles son aquellas que incorporan 6, 7 y 8 unidades de α-D-glucopiranosilo y se denominan α-, β- y γ-ciclodextrina, respectivamente (Figura 3.7.). Las modificaciones químicas controladas en estas ciclodextrinas naturales requieren el desarrollo de estrategias sintéticas eficientes que permitan modificar su estructura ya sea para mejorar sus propiedades de solubilidad en medio acuoso, la formación de complejos de inclusión o la capacidad de transporte selectivo.[42,43,44,45] Actualmente se comercializan más de 30 especialidades farmacológicas que contienen CDs en su formulación, esencialmente βCD.[46,47,48,49]

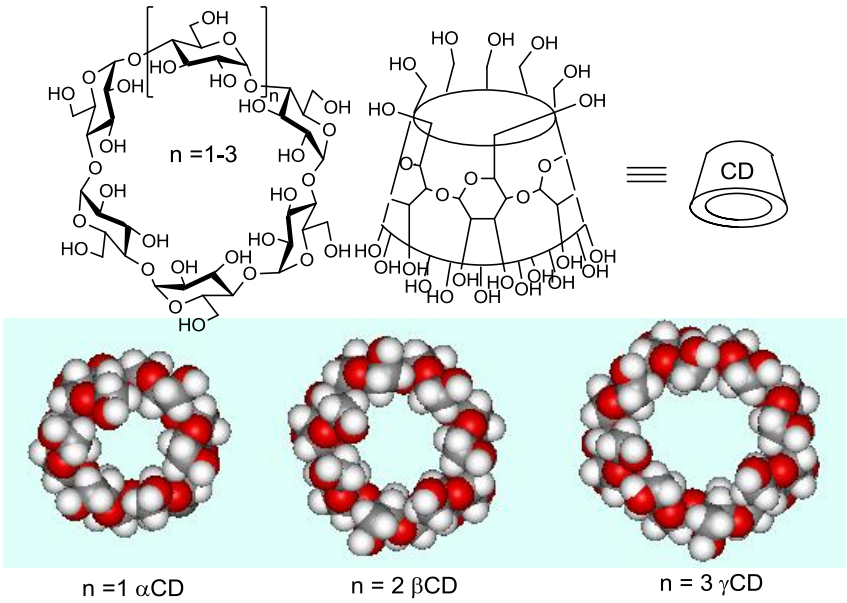


Figura 1.7. Estructura de las ciclodextrinas naturales (CDs).

Existen, no obstante, bastantes limitaciones respecto al uso farmacéutico de las CDs naturales, debido principalmente a una solubilidad en agua insuficiente de los correspondientes complejos de inclusión y, en el caso de la βCD, a su carácter hemolítico, consecuencia de su capacidad para complejar el colesterol de las membranas de los eritrocitos.[50] Otras restricciones importantes se refieren al tamaño del principio activo y a la capacidad de carga, que vienen determinados por el tamaño de la cavidad de la ciclodextrina y por la estequiometría de los complejos de inclusión (1:1 en los casos más favorables). Por otra parte, muchas aplicaciones requerirían dotar al transportador de propiedades de reconocimiento específico, capacidad de autoorganización y posibilidad de interaccionar con biomoléculas.

La incorporación de biomarcadores selectivos en las CDs que faciliten el transporte dirigido de fármacos es esencial para reducir la dosis y evitar los efectos secundarios debido a la interacción del fármaco con las células sanas. Estos sistemas

supramoleculares deben aunar una serie de características importantes para su uso farmacológico, como son solubilidad y estabilidad en agua, eficacia de vectorización, capacidad de encapsulamiento, y ausencia de carácter inmunogénico y de toxicidad. Para facilitar su aprobación por las autoridades reguladoras, deben poseer también una composición homogénea, de estructura bien definida y con un número preciso de epítopos reconocibles.[35] La implementación de métodos de funcionalización selectiva de ciclodextrinas puede explotarse para la incorporación de ligandos complementarios de receptores expresados en células o tejidos diana específicos dentro del organismo (ciclodextrinas de tercera generación).[51] Nuestro grupo de investigación tiene una amplia experiencia en este campo, habiendo propuesto varios prototipos de glicotransportadores para la vectorización y liberación controlada de fármacos. Además, dispone de técnicas adecuadas para la evaluación de interacciones entre oligosacáridos y receptores celulares.[51,52] Por otra parte, el grupo de investigación ha dedicado un esfuerzo considerable al desarrollo de chaperonas farmacológicas para el tratamiento de enfermedades de depósito lisosomal. Los mejores candidatos son glicomiméticos monosacarídicos que incorporan un fragmento hidrófobo, con una solubilidad en agua limitada. En el caso concreto de la enfermedad de Gaucher, las células más afectadas son los macrófagos. Dentro de esta línea, en esta Tesis se han diseñado sistema de transporte de chaperonas farmacológicas adaptados a estas necesidades, en concreto glicotransportadores basados en la βCD con antenas de biorreconocimiento sacarídicas complementarias de receptores celulares sobreexpresados en la membrana celular de macrófagos (Figura 1.8).[51]

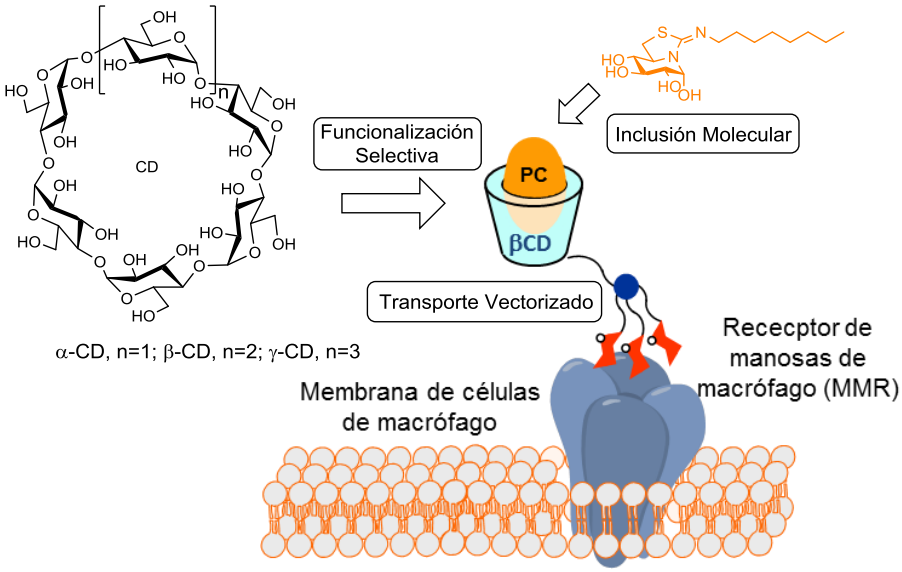


Figura 1.8. Vectorización de fármacos mediada por ciclodextrinas selectivamente funcionalizadas (CDs de tercera generación) provistas de elementos de biorreconocimiento.

Los derivados moleculares de ciclodextrinas no son adecuados para el transporte y liberación controlada de biomoléculas, como los ácidos nucleicos, dado que exceden con creces el tamaño de la cavidad. El empleo de genes como agentes terapéuticos representa una potente herramienta para tratar un amplio rango de enfermedades tanto congénitas como adquiridas.[53] El aumento de ensayos clínicos aprobados cada año muestra la creciente importancia de esta modalidad de medicina molecular (Figura 1.9.).[54]

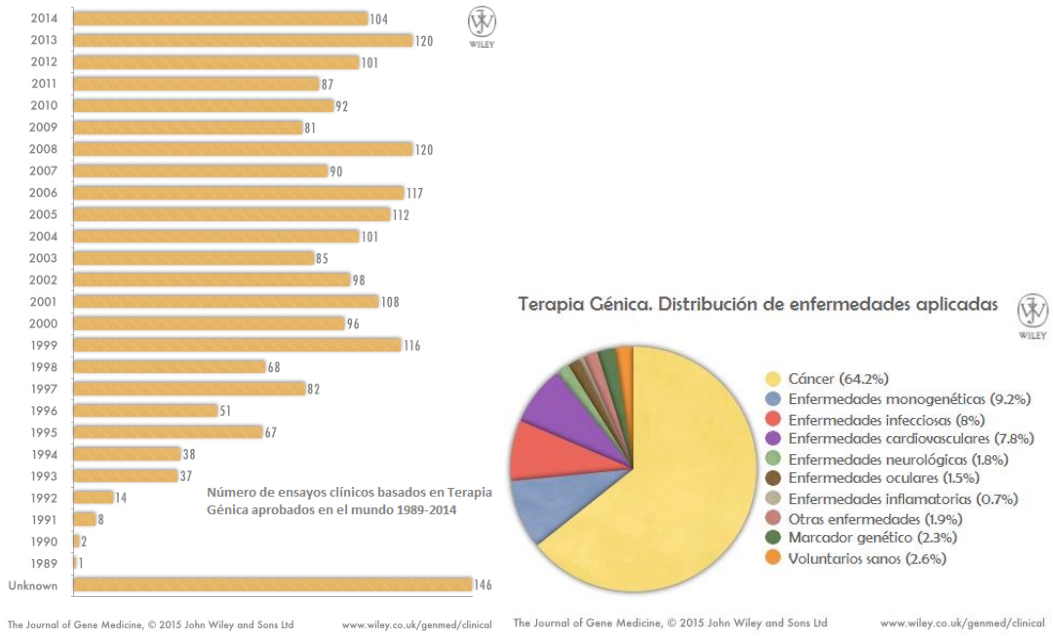


Figura 1.9. Número de ensayos clínicos con terapia génica aprobados, a nivel mundial, entre 1989 y 2014 (izquierda) y distribución de las enfermedades en las que se han aplicado (derecha).[54]

Aunque el material génico puede llegar a internalizarse y expresarse en células por sí solo –un proceso denominado *transfección*– presenta una baja estabilidad como consecuencia de su rápida degradación en los fluidos biológicos por la acción de las nucleasas así como por su interacción inespecífica con los componentes del suero samguíneo, por lo que su eficacia es muy limitada. Además, la internalización celular está restringida debido al tamaño de estas macromoléculas y a la repulsión electrostática entre los fosfolípidos de la membrana celular y los grupos fosfato de los ácido nucleicos, ambos cargados negativamente. Se trata de mecanismos de defensa que la evolución ha proporcionado a las células para impedir la intrusión de material génico foráneo. Esto hace que el uso terapéutico de ácidos nucleicos requiera el desarrollo de métodos de administración adecuados. Si bien existen técnicas físicas de transfección, como la microinyección, la transfección biobalística, la aplicación de ultrasonidos, el empleo de

gradientes de presión y la electroporación,[55] en general son agresivas y su estandarización resulta difícil, por lo que el uso de *vectores de transfección* de genes está más ampliamente extendido. Un vector de transfección es un transportador de fármacos capaz de formar una estructura supramolecular de dimensiones nanométricas con el elemento terapéutico que es un ácido nucleico, y que lo protege del entorno durante su transporte hacia la célula objetivo, liberándolo en las proximidades del núcleo para que exprese la información que porta antes de ser degradado por las nucleasas citoplasmáticas. Estos vectores pueden clasificarse en dos grandes grupos: virales y no virales. Teniendo en cuenta que los virus son sistemas naturales especialmente diseñados para transferir material génico a células, el uso de virus recombinantes, en los que se elimine su capacidad de replicación, ha sido ampliamente investigado para su aplicación en terapia génica.[56,57,58] Sin embargo, su limitada capacidad de carga de material génico, su alto coste de producción y las reservas acerca de la bioseguridad de su empleo (toxicidad, posibilidad de desarrollar respuesta inmune y/o oncogénesis) han hecho que en los últimos años, se haya intensificado la investigación en el diseño y síntesis de vectores de transfección no virales.[59] La mayoría de las formulaciones que utilizan vectores no virales para la transfección génica corresponden a dos grupos de compuestos: los lípidos catiónicos[60,61,62] y polímeros catiónicos,[63,64,65,66] sustancias capaces de formar complejos con el ADN libre llamados lipoplejos o poliplejos, respectivamente.

Los polímeros catiónicos han demostrado tener una alta eficacia de transfección alcanzando, en algunos casos, niveles de expresión comparables a vectores virales. Sin embargo, su funcionalidad está limitada por la ausencia de mecanismos para liberar el poliplejo del sistema vesicular celular.[67] La poli-L-lisina (PLL, Figura 1.10.) fue el primer polímero catiónico evaluado como vector polimérico del ADN.[68] A pesar de su eficacia de internalización celular, los niveles de expresión fueron moderados como consecuencia de la lenta liberación del endosoma por el alto grado de protonación de los grupos amino de PLL a pH fisiológico.[69] La polietilenimina (PEI, Figura 1.10.), descrita por Behr,[70] es el más conocido de los polímeros sintéticos y ha dado lugar a diversos sistemas

comerciales para transfección *in vitro*, tales como el Jet-PEI o ExGen500. Su excelente eficacia de transfección asociada a la alta densidad de grupos catiónicos se ve contrarrestada con su relativamente elevada toxicidad. El hecho de que estas formulaciones sean polidispersas y presenten propiedades conformacionales aleatorias dificulta considerablemente el estudio de correlaciones entre características estructurales, eficacia de complejación y transporte del material génico.[59]

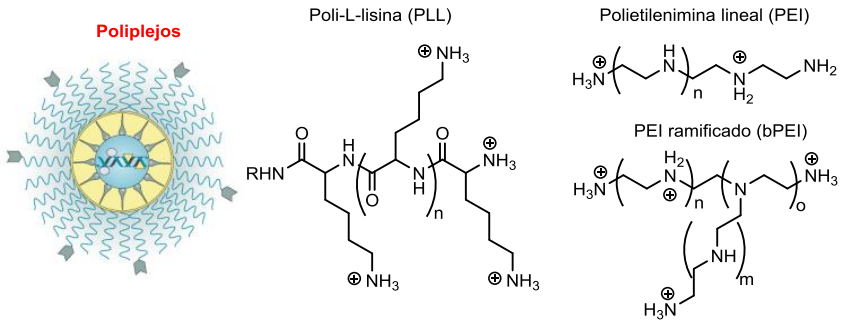


Figura 1.10. Ejemplos de polímeros catiónicos empleados como vectores de genes.

Los lípidos catiónicos son moléculas anfifílicas que contienen un grupo cargado positivamente unido a un dominio hidrófobo a través de un conector. En medio acuoso, cuando su concentración es mayor a la concentración micelar crítica (CMC), forman grandes estructuras vesiculares conocidas como *liposomas*. Éstos son capaces de formar complejos electrostáticos con los ácidos nucleicos cargados negativamente (lipoplejos), permitiendo así su paso por la membrana celular. En 1987 se empleó por primera vez, un lípido catiónico como vector de genes, el cloruro de *N*-[1-(2,3-dioleiloxi)propil]-*N*,*N*,*N*-trimetilamonio (DOTMA, Figura 1.11).[71] Desde entonces numerosos estudios de estructura-actividad han demostrado que parámetros estructurales tales como la longitud de la cadena hidrófoba, el tamaño y carga del grupo cargado, naturaleza del conector y la sensibilidad al pH juegan un papel primordial en modular la eficiencia de los lipoplejos.[72] Li y colaboradores demostraron la eficacia de transfección *in vivo* del *N*-[1-(2,3-dioleoiloxi)propil]-*N*,*N*,*N*-trimetilamonio metil sulfato (DOTAP) para el tratamiento de

enfermedades pulmonares en ratones.[73] Otro lipoplejo derivado de colesterol (DC-Chol) fue el primer lípido catiónico empleado en ensayos clínicos.[74]

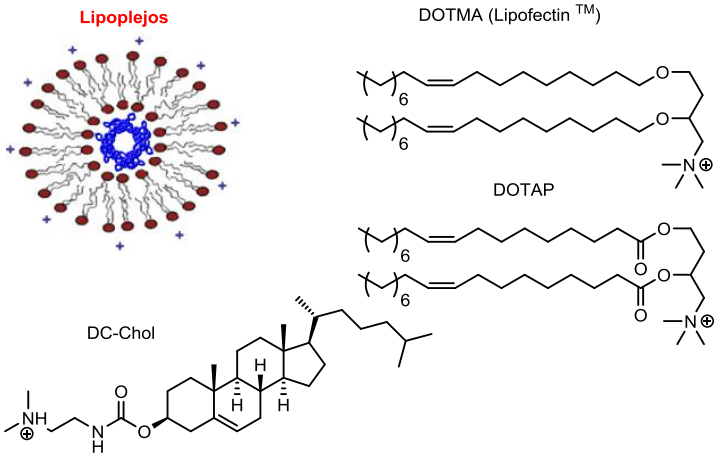
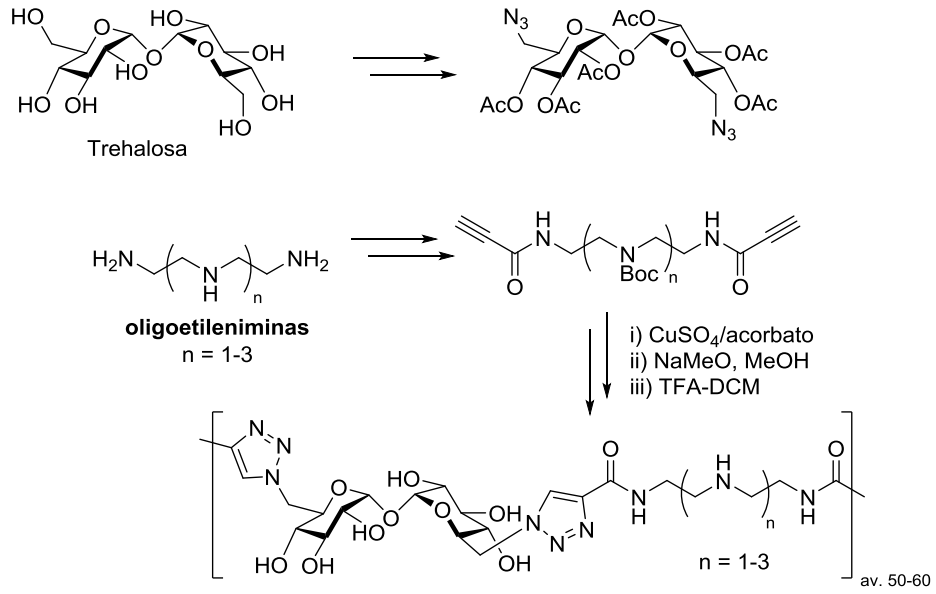


Figura 1.11. Ejemplos de lípidos catiónicos empleados como vectores de genes.

Los carbohidratos también han contribuido notablemente en este campo gracias a la diversidad estructural, biocompatibilidad y alta disponibilidad de los azúcares sencillos. La utilización de carbohidratos en el diseño de nuevos polímeros catiónicos como vectores de transfección no-virales ha demostrado ventajas significativas.[75,76,77,78] Por ejemplo, Reineke y Davis observaron que la intercalación de fragmentos sacarídicos (ej: trehalosa) entre los fragmentos catiónicos de poliamidinas disminuía la citotoxicidad de estos transportadores de genes sin afectar a su capacidad de transfección (Esquema 1.1.).[79]



Esquema 1.1. Síntesis de polímeros catiónicos basados en trehalosa-poliamidinas.

Estas ventajas atribuidas a la insersión de carbohidratos no están sólo restringidas a los vectores no-virales basados en polímeros catiónicos. También en el caso de los lípidos catiónicos se han descrito ejemplos de lipoplejos funcionalizados con carbohidratos[80] con objeto de reforzar la interacción lípido-ácidos nucleicos. En los últimos años, ha emergido una nueva familia de vectores no virales de carácter molecular, intrínsecamente monodispersos, entre los que los derivados de ciclodextrinas juegan un papel destacado.[81,82] Aunque se han propuesto numerosos ejemplos de derivados policatiónicos de ciclodextrinas como vectores de transfección no virales,[83,84,85,86,87,88] los más eficaces son aquellos derivados que presentan anfifilicidad facial,[84,89,90,91,92] esto es, constan de dos dominios diferenciados, uno consistente en un clúster policatiónico y otro formado por múltiples cadenas lipófilas que pueden estar dispuestos en las caras primaria o secundaria indistintamente (Figura 1.12. modelos A y B).

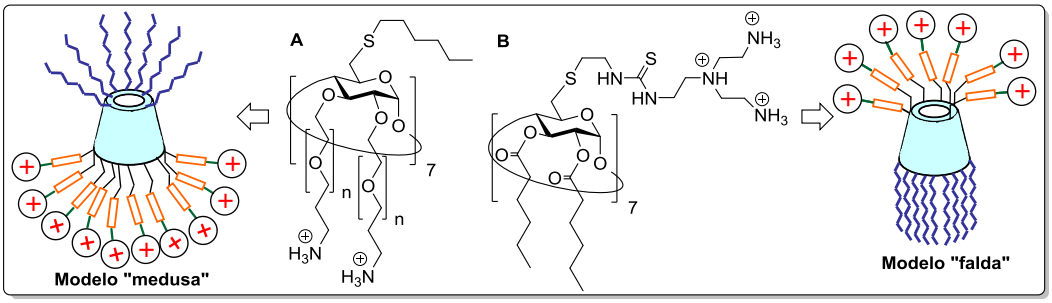


Figura 1.12. Ejemplos de ciclodextrinas anfifílicas monodispersas con orientación relativa de los dominios hidrófilo y lipófilo de tipo "medusa" (A) y "falda" (B) utilizadas como vectores de transfección.

En este contexto el desarrollo de nuevos vectores de transfección génica con estructura de lípidos catiónicos a partir de plataformas sacarídicas más simples como la metil α-D-glucosa, la α,α'-trehalosa y las ciclotrehalanas[93,94] –derivados cíclicos diméricos de trehalosa unidos por puentes de tiourea– parece un prometedor campo de investigación. Estas plataformas sacarídicas presentan a priori las ventajas inherentes a los glicolípidos catiónicos: biocompatibilidad, biodegradabilidad, carácter monodisperso y anfifilicidad facial –esto último sobre todo en el caso de los derivados de trehalosa y ciclotrehalanas. Adicionalmente, la funcionalización selectiva de estas plataformas para la obtención de variabilidad estructural es más sencilla y eficiente que en las ciclodextrinas. Es por ello que uno de los objetivos de esta Tesis se ha centrado en explorar la viabilidad de estos derivados anfifílicos sacarídicos como glicotransportadores de genes mediante estudios de sus propiedades de autoorganización, de complejación y protección de ADN, y estudios de su capacidad de transfección celular.

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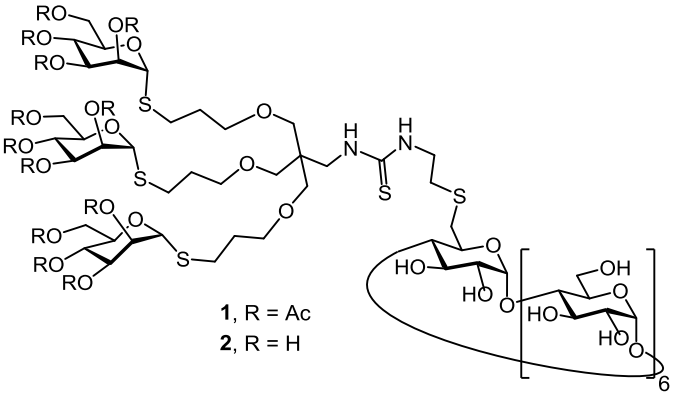
Chapter 2
Objetivos

2. Objetivos.

El desarrollo de los capítulos de esta Tesis ha dado lugar a la preparación de una serie de nuevos compuestos y precursores cuyas estructuras se presentan a continuación:

Transporte vectorizado de chaperonas farmacológicas a macrófagos



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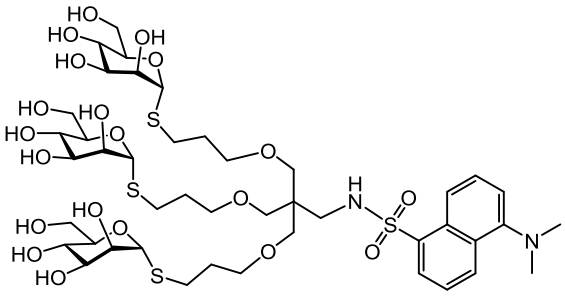
2, R = H



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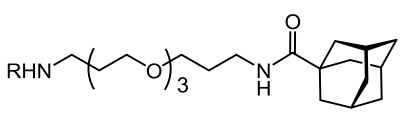
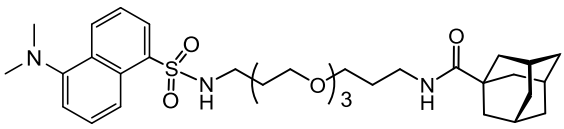
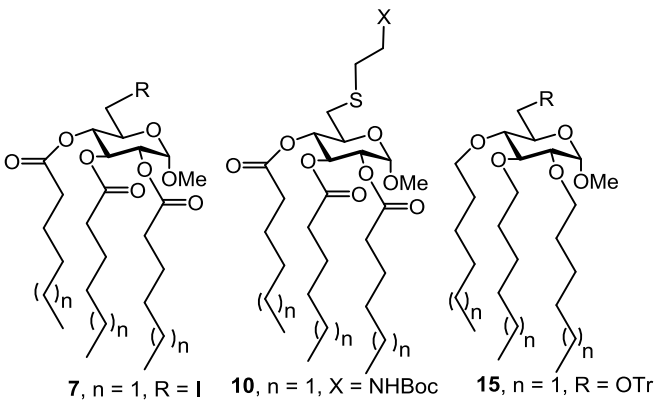


4, R = Boc
5, R = H



6

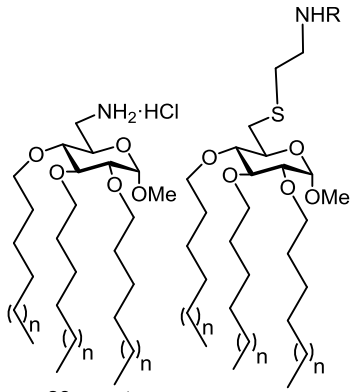
Derivados catiónicos anfífilicos de glucosa y trehalosa.



7, n = 1, R = I
8, n = 9, R = I
9, n = 1, R = N_3

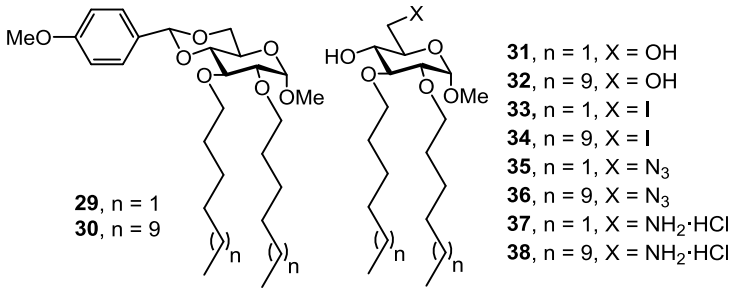
10, n = 1, X = NHBoc
11, n = 1, X = NH_2·HCl
12, n = 9, X = NHBoc
13, n = 9, X = NH_2·HCl
14, n = 1, X = N_3

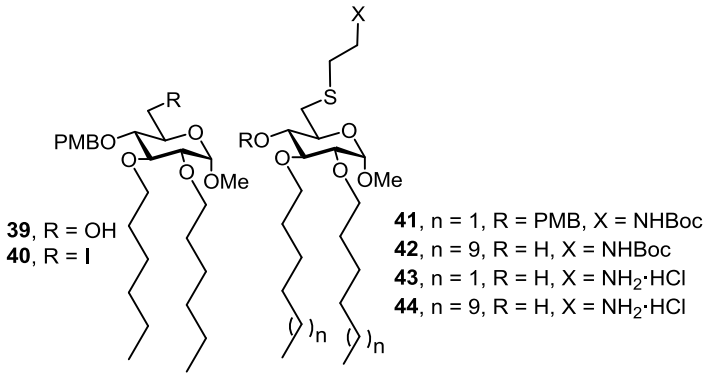
15, n = 1, R = OTr
16, n = 1, R = OH
17, n = 9, R = OTr
18, n = 9, R = OH
19, n = 1, R = I
20, n = 9, R = I
21, n = 1, R= N_3
22, n = 9, R = N_3

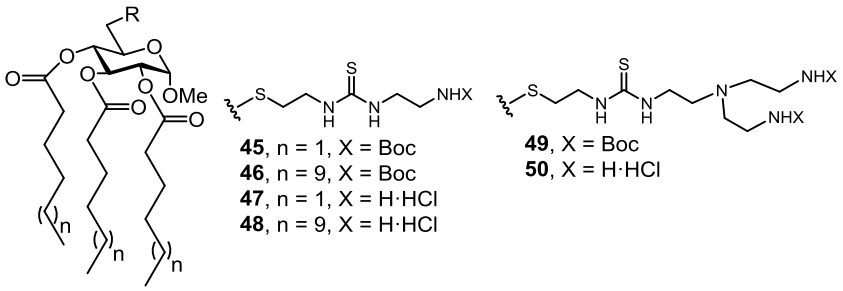


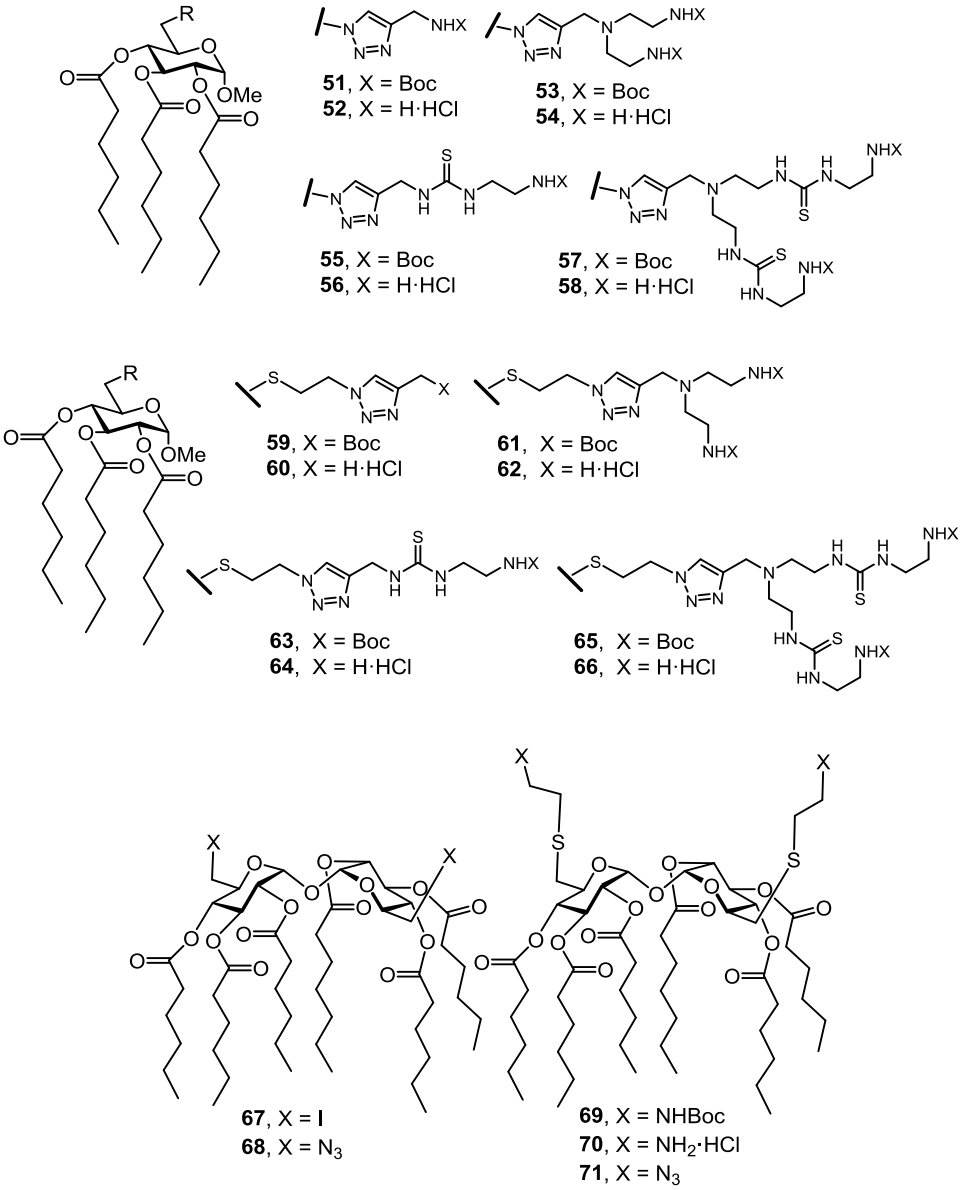
23, n = 1
24, n = 9

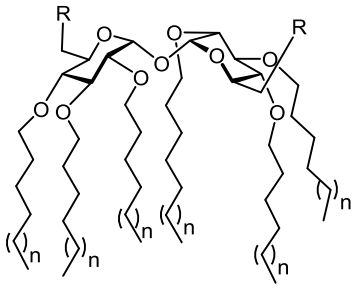
25, n = 1, R = Boc
26, n = 9, R = Boc
27, n = 1, R = H·HCl
28, n = 9, R = H·HCl


MeO
OMe
HO
X
29, n = 1
30, n = 9
31, n = 1, X = OH
32, n = 9, X = OH
33, n = 1, X = **I**
34, n = 9, X = **I**
35, n = 1, X = N_3
36, n = 9, X = N_3
37, n = 1, X = NH_2·HCl
38, n = 9, X = NH_2·HCl

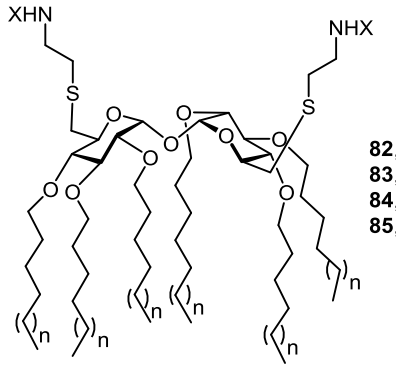

PMBO
RO
R
X
S
OMe
39, R = OH
40, R = **I**
41, n = 1, R = PMB, X = NHBoc
42, n = 9, R = H, X = NHBoc
43, n = 1, R = H, X = NH_2·HCl
44, n = 9, R = H, X = NH_2·HCl


R
OMe
S
NHX
45, n = 1, X = Boc
46, n = 9, X = Boc
47, n = 1, X = H·HCl
48, n = 9, X = H·HCl
49, X = Boc
50, X = H·HCl

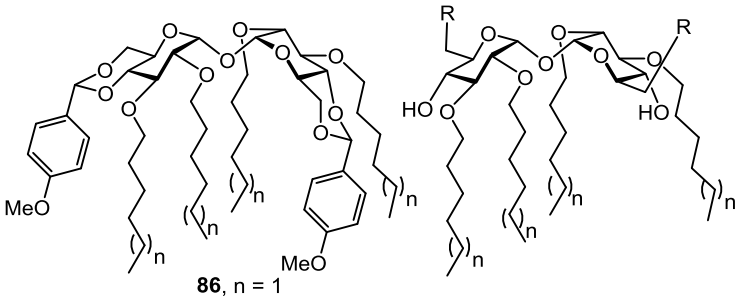

R
OMe
NHX
51, X = Boc
52, X = H·HCl
53, X = Boc
54, X = H·HCl
55, X = Boc
56, X = H·HCl
57, X = Boc
58, X = H·HCl
59, X = Boc
60, X = H·HCl
61, X = Boc
62, X = H·HCl
63, X = Boc
64, X = H·HCl
65, X = Boc
66, X = H·HCl
67, X = I
68, X = N3
69, X = NHBoc
70, X = NH2·HCl
71, X = N3



72, n = 1, R = OTr
73, n = 9, R = OTr
74, n = 1, R = OH
75, n = 9, R = OH
76, n = 1, R = **I**
77, n = 9, R = **I**
78, n = 1, R = N_3
79, n = 9, R = N_3
80, n = 1, R = NH_2·HCl
81, n = 9, R = NH_2·HCl

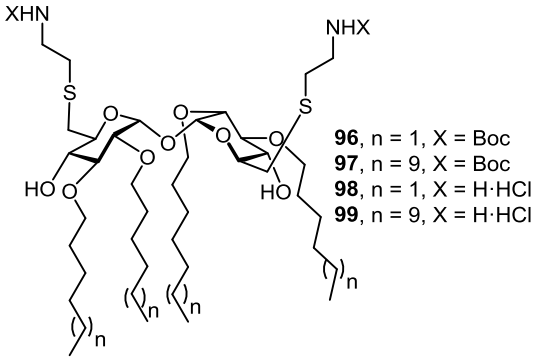


82, n = 1, X = Boc
83, n = 9, X = Boc
84, n = 1, X = H·HCl
85, n = 9, X = H·HCl

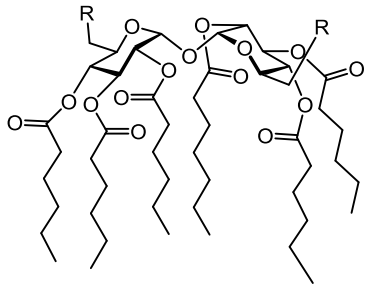


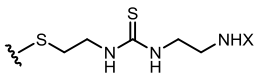
86, n = 1
87, n = 9

88, n = 1, R = OH
89, n = 9, R = OH
90, n = 1, R = **I**
91, n = 9, R = **I**
92, n = 1, R = N_3
93, n = 9, R = N_3
94, n = 1, R = NH_2·HCl
95, n = 9, R= NH_2·HCl

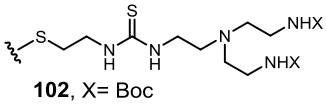


96, n = 1, X = Boc
97, n = 9, X = Boc
98, n = 1, X = H·HCl
99, n = 9, X = H·HCl

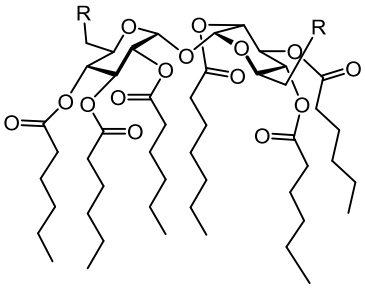


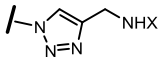


100, X = Boc
101, X = H

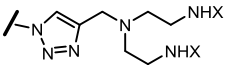


102, X= Boc
103, X= H·HCl





104, X = Boc
105, X = H·HCl



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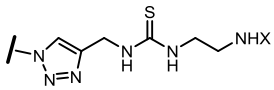
107, X = H·HCl



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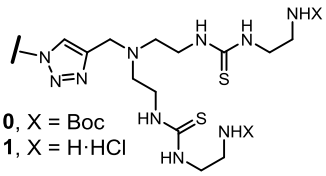
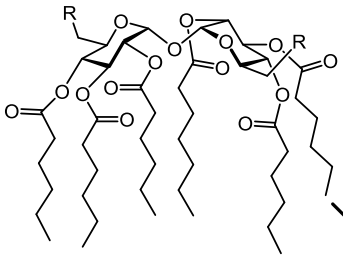
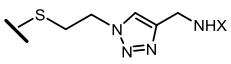
109, X = H·HCl



110, X = Boc
111, X = H·HCl





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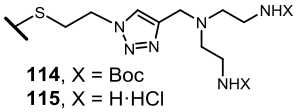

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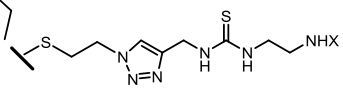

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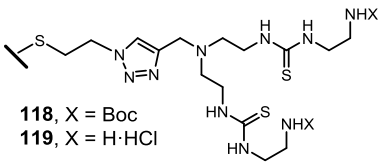
117, X = H·HCl



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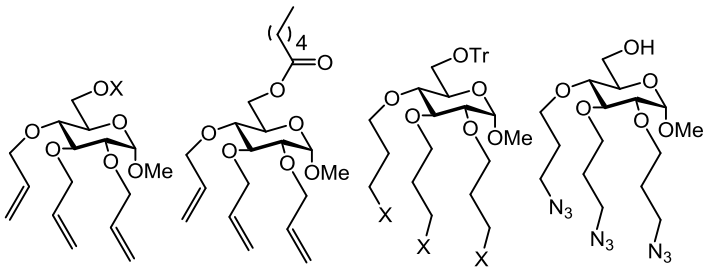
119, X = H·HCl

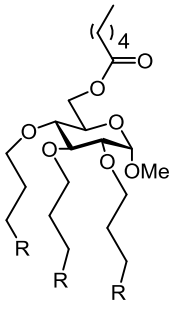


120, X = Tr
121, X = H

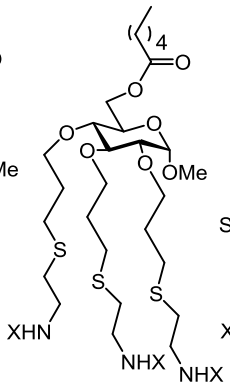
122

123, X = OH
124, X = OMs
125, X = N_3

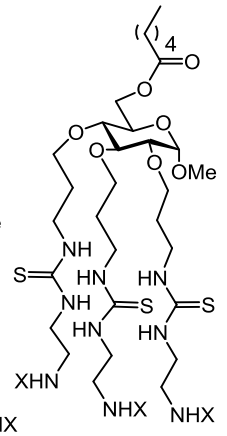
126



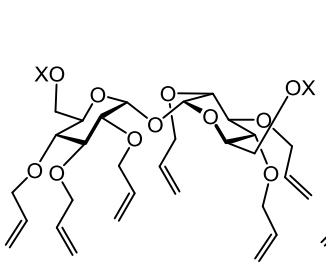
127, R = N_3
128, R = NH_2·HCl



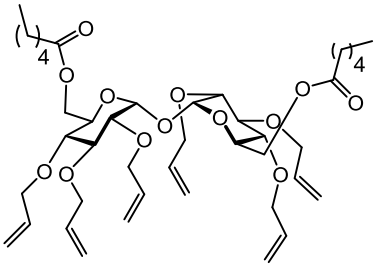
129, X = Boc
130, X = H·HCl



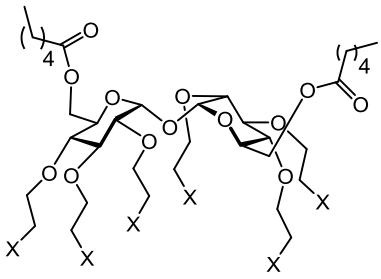
131, X = Boc
132, X = H·HCl



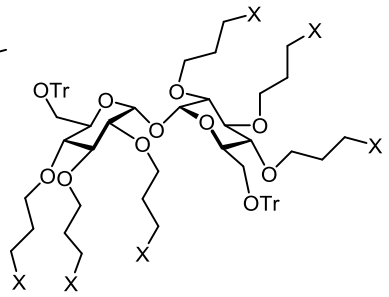
133, X = Tr
134, X = H



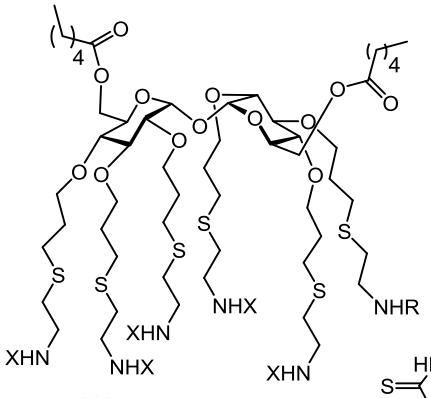
135



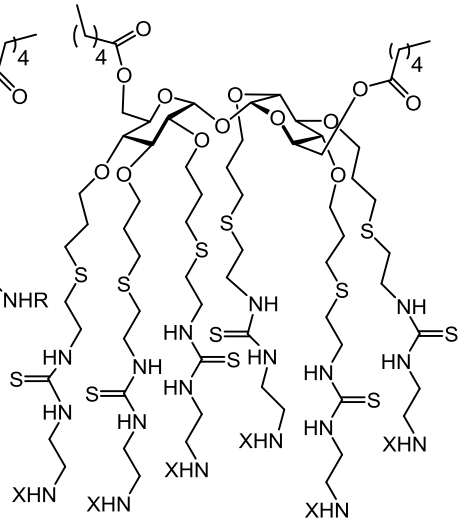
136, X = N_3
137, X = NH_2.HCl



138, X = OH
139, X = OMs
140, X = N_3

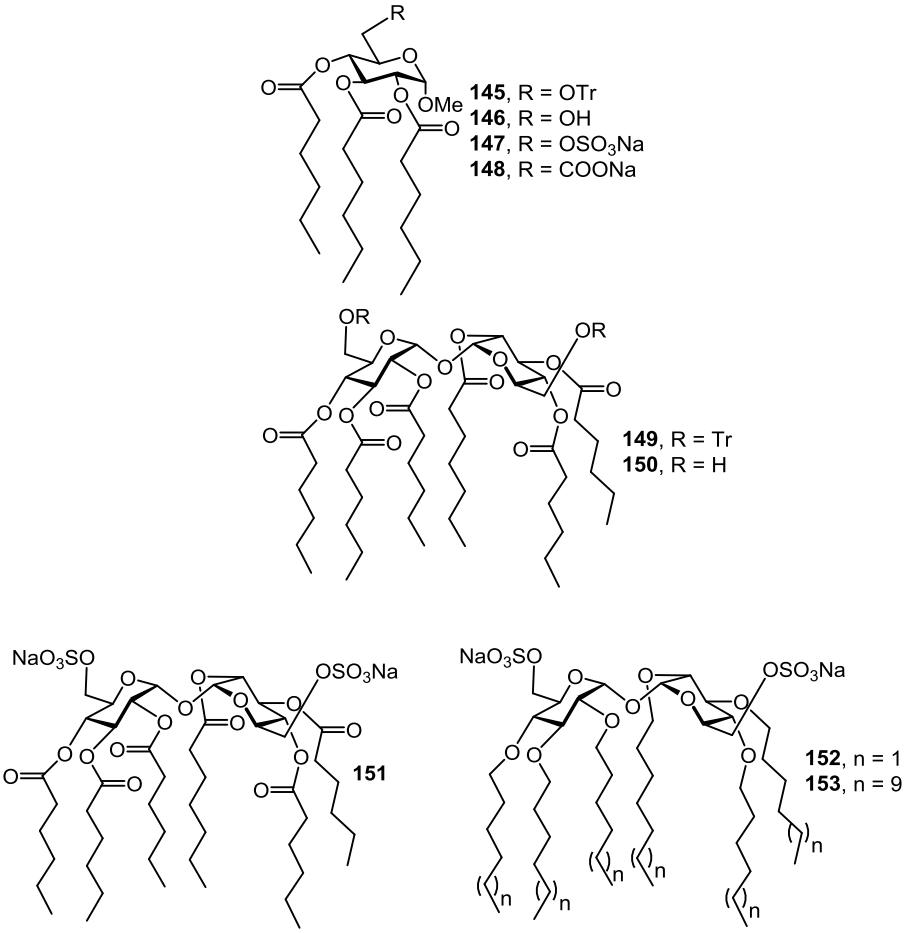


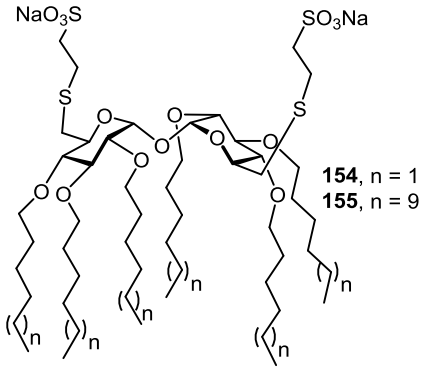
141, X = Boc
142, X = H·HCl



143, X = Boc
144, X = H·HCl

Derivados aniónicos anfifílicos de glucosa y trehalosa.

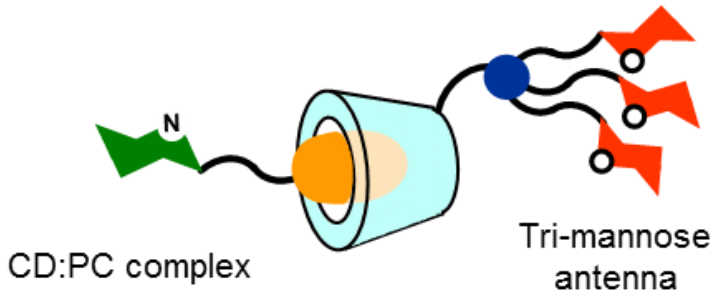



NaO3S
SO3Na
S
S
O
O
O

Capítulo 3

Transporte vectorizado de chaperonas farmacológicas a macrófagos para el tratamiento de la enfermedad de Gaucher.

Abstract: Pharmacological chaperone therapy (PTC) is a promising tool for the treatment of a wide number of protein folding disorders, among which the Gaucher disease presents the highest prevalence. Amphiphilic sp^2-iminosugars based in D-gluco or D-galacto hydroxylation pattern, carrying a hydrophobic substituent, have been found to be useful in restoring GCase activity, presenting pharmacological chaperone (PC) behaviour. Unfortunately, these amphipatic molecules have very low water solubility, strongly limiting their bio-availability. This problem could be partially solved by a co-formulation with β-cyclodextrin (βCD), that can form a stable inclusion complex with amphiphilic sp^2-iminosugars and deliver the chaperone to the active site of Gcase. But, in addition, this molecules lack any structural motif to enable specific cell targeting. Herein, we designed a targeted delivery system based on a β-CD core, by implementing a strategy for the selective functionalization of the βCD with a mannomultivalent recognition antenna.



3. Transporte vectorizado de chaperonas farmacológicas a macrófagos para el tratamiento de la enfermedad de Gaucher

3.1. Introducción

Las ciclodextrinas y sus derivados con modificaciones químicas se están convirtiendo en importantes herramientas en el campo farmacéutico. Debido a su estructura toroidal bien definida, existe una diferencia de reactividad química entre la cara primaria (compuesta por 7 hidroxilos primarios libres en el caso de la βCD) y la cara secundaria (compuesta por 14 hidroxilos secundarios libres en el caso de la βCD). Las estrategias puestas a punto en el grupo de investigación permiten la funcionalización selectiva de la cara primaria de la βCD y la incorporación de ligandos biorreconocibles con una orientación espacial definida organizados en un entorno simétrico de dimensiones nanométricas. Este concepto ha sido aplicado a la preparación de glicoconjugados hiperramificados que se comportan como modelos de glicocálix celular en su interacción con lectinas específicas.[1]

Por otro lado, el grupo de investigación presenta una amplia experiencia en la síntesis y evaluación de inhibidores específicos de glicosidasas. La amplia gama de procesos fisiológicos y patológicos en los que intervienen dichas enzimas ha estimulado una intensa investigación dirigida a identificar inhibidores específicos de las mismas, tanto procedentes de fuentes naturales como sintéticos.[2] Entre los inhibidores reversibles de glicosidasas, los análogos de monosacáridos (glicomiméticos) en los que el oxígeno endocíclico ha sido sustituido por un átomo de nitrógeno (iminoazúcares) se encuentran entre los más versátiles, siendo ampliamente utilizados en investigación tanto básica como aplicada en glicobiología (Figura 3.1.).[3] En la naturaleza se encuentran miméticos de monosacáridos con estructura de iminoazúcar que actúan como inhibidores potentes de glicosidasas de amplio espectro, es decir, inhiben simultáneamente varias glicosidasas.[2]

Frecuentemente pueden inhibir varias α y β-glicosidasas y esta falta de selectividad anomérica representa un serio obstáculo para el desarrollo de aplicaciones clínicas.

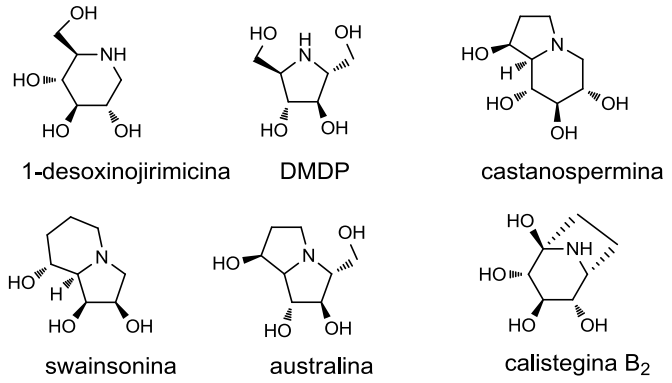


Figura 3.1. Estructuras de los iminoazúcares naturales polihidroxilados más representativos.

El concepto de ‘’*sp*^{2}-iminoazúcares’’ ha permitido desarrollar nuevos glicomiméticos en los que el nitrógeno imínico *sp*^{3} pose ha sustituidor un átomo de nitrógeno de tipo pseudoamida (tiourea, urea, isourea, isotiourea, carbamato, tiocarbamato o guanidina) con un importante carácter *sp*^{2}, que se comportan como inhibidores de glicosidasas con una elevada selectividad anomérica (Figura 3.2.).[4] Este cambio estructural aumenta la contribución orbitálica al efecto anomérico en el centro aminoacetálico, permitiendo acceder a *sp*^{2}-iminoazúcares estables, reductores o no reductores, que muestran integridad conformacional y configuracional en agua, a diferencia de lo que sucede con los iminoazúcares clásicos. Es importante destacar que este rasgo estereoelectrónico está presente en algunos inhibidores de origen natural como la trehazolina, un potente inhibidor de trehalasa.[5]

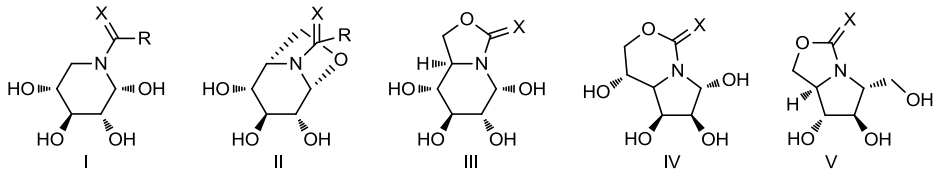


Figura 3.2. Estructura de algunos tipos de *sp*^{2}-iminoazúcares (X = O, S, NHR).

Por otro lado, bajo el término ‘’enfermedades de almacenamiento lisosomal’’ (**Lysosomal Storage Diseases, LSDs**) se encuentran más de 40 desórdenes genéticos caracterizados por la disfunción de enzimas implicadas en la degradación metabólica de determinados glicoconjugados.[6] La deficiencia enzimática conduce a la acumulación de sustancias tóxicas en los macrófagos, con consecuencias degenerativas e incluso letales. En la actualidad se están estudiando diferentes terapias para el tratamiento de estas enfermedades.[7] Recientemente se ha desarrollado una nueva terapia, denominada de “acompañante químico” donde se ha demostrado que inhibidores de glicosidasas potentes y selectivos son capaces de inducir el plegamiento correcto del enzima mutado, que en estas condiciones recupera su estabilidad, siendo transportado hasta el lisosoma donde degrada al sustrato.[8,9] Un factor limitante asociado a estos tratamientos es la baja biodisponibilidad de los agentes terapéuticos y, por tanto, la dificultad para alcanzar la concentración necesaria para su actividad.[10] El desarrollo de fármacos viables, incluso para estructuras que muestren grados de actividad elevados en evaluaciones *in-vitro* y *ex-vivo*, requeriría solucionar aspectos relativos a la solubilidad y estabilidad de los inhibidores en el medio biológico y a la posibilidad de conducirlos selectivamente a células o tejidos específicos. Además del deterioro neurológico, uno de los problemas que conllevan estas enfermedades es la visceromegalia, especialmente de hígado y bazo. Por ello, un aspecto importante a considerar es el desarrollo de inhibidores de glicosidasas dotados de la capacidad de reconocimiento a nivel de receptores celulares, a través de una estrategia supramolecular, basada en la inclusión de la parte hidrófoba en derivados de ciclodextrinas (Figura 3.3.).[11]

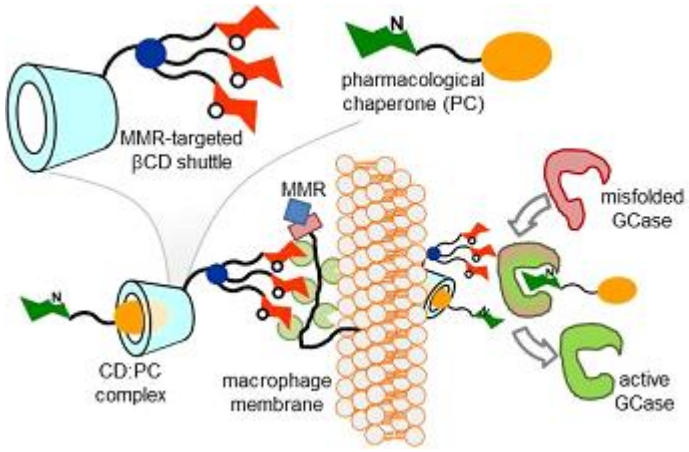


Figura 3.3. Vectorización de fármacos mediada por ciclodextrinas selectivamente funcionalizadas (CDs de tercera generación) provistas de elementos de biorreconocimiento.

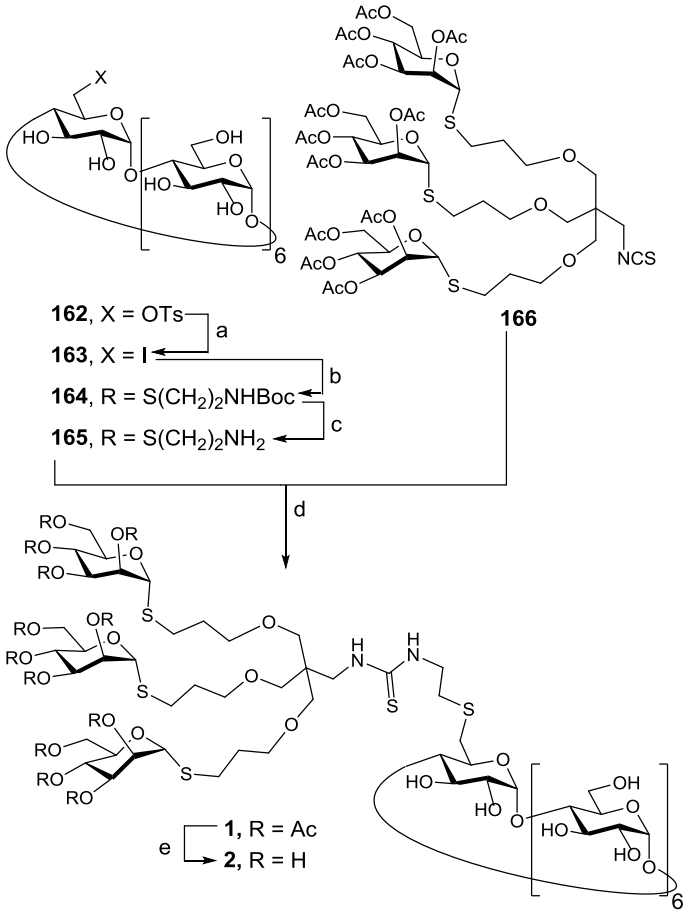
3.2. Resultados y Discusión

3.2.1. Criterios de selección de las chaperonas farmacológicas y diseño de un conjugado trimanosilado de β-ciclodextrina ((ManS)$_3$-βCD) como transportador.

Los derivados bicíclicos con estructura de 5*N*,6*S*-(*N'*-alquiliminometiliden)-6-tionojirimicina se comportan como inhibidores potentes y selectivos frente a varias β-glucosidasas presentando además excelentes propiedades como chaperonas farmacológicas frente a mutaciones asociadas a la GCasa.[12] Para este estudio hemos seleccionado los derivados de *N*'-octilo y *N'*-[4-(adamant-1-ilcarboxamido)butil] **6S-NOI-NJ**[13] y **6S-NAdB-NJ**[14] ya que las cadenas de octilo y adamantilo muestran gran capacidad para formar complejos con la βCD en medio acuoso con valores de constantes de asociación[15] (K_{as}) en el rango de 10^2-10^4 M^{-1} por lo que cabe esperar que la formación de complejos de inclusión con un transportador basado en CD favorezca una transferencia eficaz de la PC desde la cavidad de la CD al sitio activo de la enzima. Por otra parte, como transportador adecuado de las PCs a los macrófagos hemos preparado el derivado trimanosilado (ManS)$_3$-βCD, teniendo en cuenta que en los procesos de reconocimiento

carbohidrato-proteína la multivalencia es generalmente un prerrequisito para obtener una afinidad biologicamente útil[16] y que la monosustitución del receptor de βCD con un dendrón manosilado debe alterar menos las propiedades de inclusión de un huésped que las estrategias de polisustitución.[17]

La síntesis del derivado manosilado $(ManS)_3$-βCD se ha llevado a cabo empleando una estrategia convergente que aprovecha la eficacia de la reacción de formación de tiourea para la conjugación con la macromolécula[18] (Esquema 3.1). La βCD comercial se tosiló regioselectivamente en una única posición O-6 primaria mediante reacción con cloruro de *p*-toluénsulfonilo en medio básico acuoso y en presencia de cobre (II) (→ **162**).[19] A continuación se incorporó un espaciador de cisteamina para evitar problemas de impedimento estérico durante la etapa final de conjugación y para garantizar la accesibilidad del glicoligando en los procesos de reconocimiento molecular. Sin embargo, el desplazamiento del grupo tosilato de **162** por la *N*-Boc-cisteamina requirió condiciones demasiado enérgicas y resultó problemática por lo que se sustituyó el grupo tosilato por yodo mediante tratamiento con NaI. El yoduro resultante **163** se hizo reaccionar con *N*-Boc- cisteamina para dar el aducto **164**, que tras desprotección en medio ácido del grupo carbamato condujo a la monocisteamina βCD **165**. El acoplamiento de **165** y el dendrón manosilado funcionalizado como isotiocianato **166**[20] se llevó a cabo en DMF bajo catálisis de Et_3N y permitió aislar la correspondiente tiourea **1**. La desacetilación final condujo a la molécula objetivo **2**, cuya pureza y estructura se confirmaron por espectrometría de masas, RMN y análisis elemental. La presencia de la tiourea se confirmó mediante ^{13}C RMN por la señal a 183-180 ppm (δ_{CS}) y el ensanchamiento característico de las señales asociado a la rotación restringida alrededor del enlace pseudoamida NH-(C=S) a temperatura ambiente.[21] Debido a la naturaleza asimétrica del ciclooligosacárido se produjo un solapamiento considerable de las señales en el espectro de ^1H RMN que pudo



Esquema 3.1. Preparación del transportador trimanosilado $(ManS)_3$-βCD **2.** Reactivos y condiciones: (a) NaI, acetona, cuantitativo; (b) N-Boc-cisteamina, Cs_2CO_3, DMF, 50 ºC, 67%; (c) TFA-H_2O, cuantitativo; (d) DMF, Et_3N, t.a., 50%; (e) NaOMe, cuantitativo.

3.2.2. *Estudio termodinámico de la formación de complejos de inclusión $(ManS)_3$-βCD:chaperona farmacológica por RMN.*

Para medir la afinidad de las chaperonas farmacológicas **6S-NOI-NJ** y **6S-NAdB-NJ** por el receptor **2**, se llevaron a cabo experimentos de valoración mediante ^{1}H RMN en

D_2O. Las señales que más se modificaron fueron las de los protones H-3 y H-5 de la βCD confirmando la inclusión de los residuos hidrofóbicos de las chaperonas en la cavidad de la βCD. Haciendo uso del método de variaciones continuas y un procedimiento de ajuste por mínimos cuadrados[22] se determinaron unos valores de las constantes de asociación (K_{as}) de 399 ± 4 y 1019 ± 196 M^{-1}. Las isotermas de valoración fueron compatibles con una estequiometría 1:1 en los dos casos (Figura 3.4.).

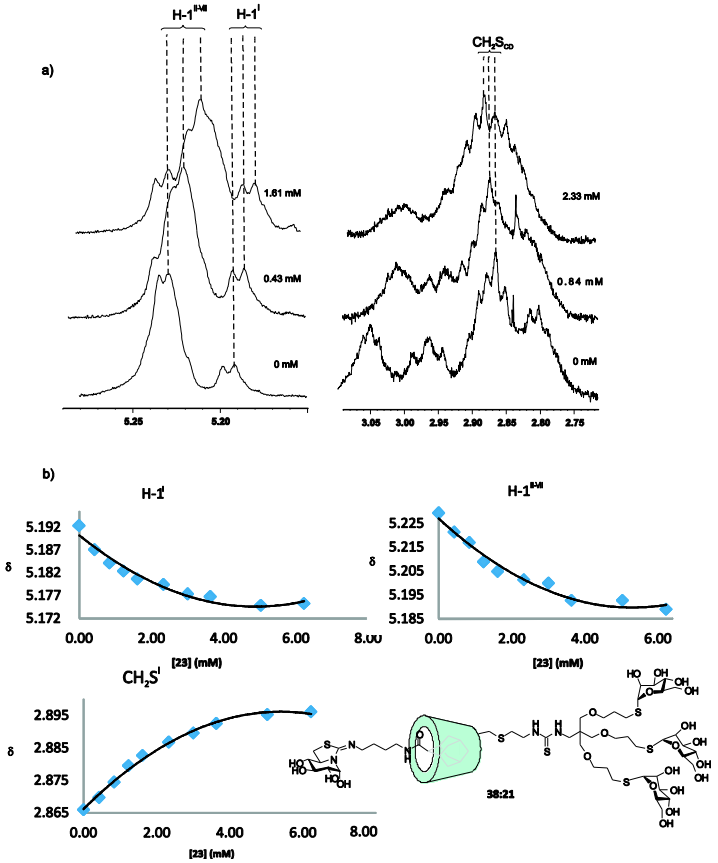


Figura 3.4. (a) Espectros de ^{1}H RMN (región anomérica) de **2** a concentraciones crecientes de **6S-NAdB-NJ**; (b) gráficas de las variaciones de δ_{H-1} frente a concentraciones crecientes de **6S-NAdB-NJ**.

3.2.3. Propiedades de inhibición de glicosidasas de los complejos (ManS)$_3$-βCD:chaperonas farmacológica.

Con objeto de confirmar si la complejación de las chaperonas farmacológicas con **2** afecta a la interacción con glicosidasas, se evaluaron las propiedades de **6S-NOI-NJ** y **6S-NAdB-NJ** y de sus complejos con **2** frente a dos β-glucosidasas (β-Glcasas) comerciales, la β-Glcasa de almendras y la β-Glcasa de hígado bovino, que pertenecen al mismo grupo que la GCasa humana (clan A).[23] Los complejos se prepararon por liofilización de cantidades equimoleculares de cada iminoazúcar sp^2 y del transportador manosilado **2** en agua. Los valores de las correspondientes constantes de inhibición al pH óptimo de cada glicosidasa se recogen en la Tabla 3.1. El modo de inhibición, determinado mediante las gráficas Lineweaver-Burk, fue de tipo competitivo en todos los casos.

Tabla 3.1. Constantes de inhibición (K_i, μM) para **6S-NOI-NJ** y **6S-NAdB-NJ** y los correspondientes complejos 1:1 con **2** frente a glicosidasas comerciales.

Glucosidasa (pH)[a]	**6S-NOI-NJ**	**6S-NOI-NJ**: **2**	**6S-NAdB-NJ**	**6S-NAdB-NJ**: **2**
β-Glcasa (almendras, 7.3)	0.76 ± 0.05	0.29 ± 0.02	0.45 ± 0.03	5.8 ± 0.05
β-Glcasa (hígado de buey, 7.3)	3.7 ± 0.1	9.9 ± 0.1	68 ± 2.0	33 ± 0.02

[a] Los valores de K_i se determinaron a partir de las gráficas de Lineweaver-Burk.

La capacidad de inhibición de las chaperonas farmacológicas no se alteró significativamente después de la formación de los complejos, mostrando valores de K_i en el rango μm, 2-3 órdenes de magnitud más bajos que las constantes de disociación de los complejo **6S-NOI-NJ**:**2** o **6S-NAdB-NJ**:**2**.

Además, el porcentaje de inhibición enzimática para una concentración constante de iminoazúcar **6S-NOI-NJ** o **6S-NAdB-NJ** no se afectó a concentraciones crecientes de **2** hasta una relación 1:10. Estos resultados son acordes con la existencia de un equilibrio rápido que permite la transferencia eficaz de las chaperonas **6S-NOI-NJ** y **6S-NAdB-NJ** desde la cavidad de la CD hasta el sitio activo de la glicosidasa. Experimentos posteriores con la GCasa humana confirmaron esta hipótesis. En la Figura 3.5. se muestra cómo los

sp^2-iminoazúcares y sus complejos con **2** fueron igualmente eficaces en la inhibición de la GCasa usando lisados celulares a concentraciones superiores a 0.1 mM.

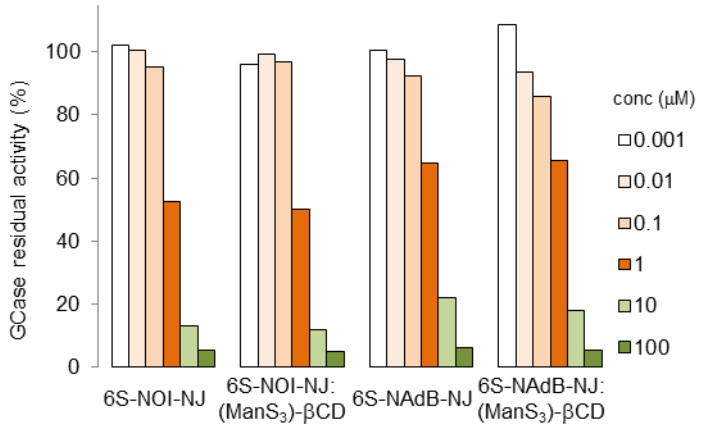


Figura 3.5. Actividad de la GCasa humana a concentraciones crecientes de las PCs **6S-NOI-NJ** y **6S-NAdB-NJ** y de sus complejos 1:1 con **2** en lisados celulares.

3.2.4. Estudio termodinámico de la formación de complejos (ManS)$_3$-βCD:chaperona farmacológica:concanavalina A por ITC.

Con objeto de confirmar la capacidad del transportador manosilado **2** y de sus complejos con las chaperonas **6S-NOI-NJ** y **6S-NAdB-NJ** para interaccionar con lectinas específicas de manosa, hemos llevado a cabo un estudio termodinámico mediante valoración calorimétrica isotérmica (ITC) frente a la lectina Concanavalina A (Con A), que reconoce específicamente a α-D-manopiranósidos.[24] En los experimentos se valoraron el transportador y sus complejos de inclusión 1:1 con las PCs frente a la Con A a pH 7.4. La estequiometría (*n*) de los complejos en todas las especies estudiadas fue 1:1 (*n* = 1); lo que indica que el dendrón manosilado (ManS)$_3$-βCD interacciona con una unidad de Con A, independientemente de si está complejado o no con la PC. La inclusión de la PC en la cavidad de la βCD no afectó de modo significativo a la afinidad por la lectinas. Los valores de las constantes de disociación (K_D) para los complejos Con A:[(ManS)$_3$-βCD:**6S-NOI-NJ**] y Con A:[(ManS)$_3$-βCD:**6S-NAdB-NJ**] se encuentran en

el rango 1.6-3.6 μM (Figura 3.6. y Tabla 3.2) comparables al valor de 2.1 ± 0.7 μm para el complejo Con A:$(ManS)_3$-βCD. Estos valores indican unos aumentos de afinidad de 20 a 80 veces en comparación a los datos publicados para el metil α-D-manopiranósido (K_D = 83 μm), empleando la misma técnica.[25] En resumen, los datos indican que el ligando manosilado trivalente del transportador**2**es capaz de intervenir en el reconocimiento de lectinas, beneficiándose del efecto multivalente y que la interacción carbohidrato-lectina es igualmente eficaz después de la formación de complejo con la PC.

Tabla 3.2. Parámetros termodinámicos y constantes de disociación (K_D) calculadas a partir de los experimentos ITC para la unión de la Con A con **2** y a los correspondientes complejos con las chaperonas **6S-NOI-NJ** y **6S-NAdB-NJ**.

	ΔG° (kJ·mol^{-1})	ΔH° (kJ·mol^{-1})	TΔG° (kJ·mol^{-1})	K_D (μM)
2	-32.4 ± 1.0	-137.7 ± 10.5	-105.2 ± 10.5	2.1 ± 0.7
6S-NOI-NJ:2	-33.0 ± 3.1	-125.3 ± 10.9	-92.3 ± 10.9	1.6 ± 0.1
6S-NAdB-NJ:2	-31.0 ± 1.3	-105.8 ± 2.4	-74.7 ± 2.4	3.6 ± 1.5

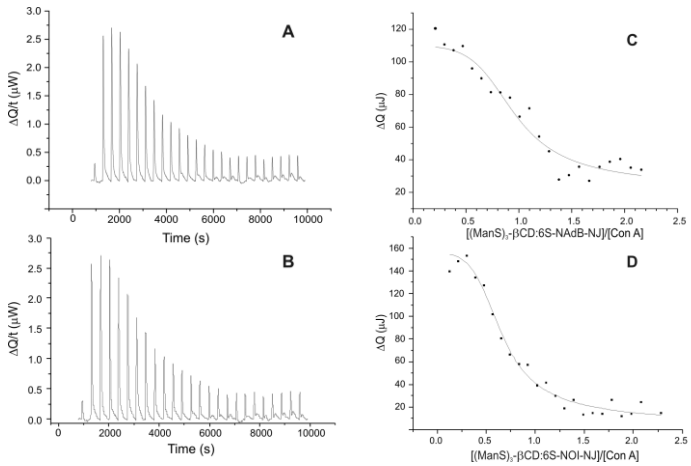


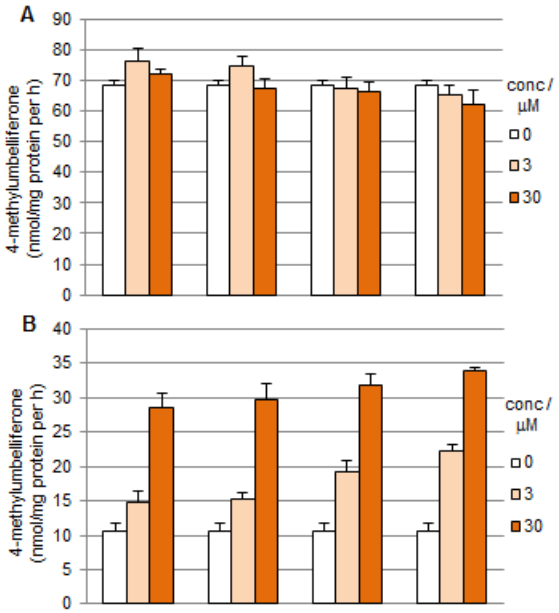
Figura 3.6. Termogramas de asociación de la Con A con los complejos 1:1 **2**:**6S-NAdB-NJ** y **2**:**6S-NOI-NJ** (A y B, respectivamente) y ajustes de mínimos cuadrados para un modelo de estequiometría 1:1 (C y D, respectivamente). ΔQ representa el calor producido tras cada inyección.

Las lectinas que reconocen un epítopo idéntico pueden ser estructuralmente muy diferentes por lo que la extrapolación de datos de su respuesta hacia la presentación multivalente de un epítopo debe tomarse con precaución. En este estudio hemos usado ensayos tipo ELLA (enzyme-linked lectin assays), empleando la lectina vegetal Con A[26] y el receptor recombinante de manosa de macrófago humano (rhMMR),[27] con objeto de validar los resultados. En el caso de la Con A se observó un incremento de afinidad de 15-veces para la **2** en comparación con el metil α-D-manopiranósido de referencia, a partir de la relación entre las concentraciones necesarias para reducir un 50% la afinidad de la Con A marcada con peroxidasa de rábano picante hacia un manano inmovilizado (IC_{50}), de acuerdo con los datos descritos en la bibliografía para otros manósidos trivalentes.[1,20,28] En el caso del receptor rhMMR, el ensayo ELLA indicó un incremento significativamente mayor (72-veces) para la **2** en comparación con el metil α-D-manopiranósido, lo que sugiere que la disposición de las unidades de manosa en el dendrón es particularmente apropiada para beneficiarse del efecto multivalente en el caso de esta lectina. De hecho, experimentos control con oligosacáridos de manosa, los ligandos naturales preferidos para este receptor muestran una afinidad análoga a la de la **2** hacia esta lectina.[28]

3.2.5. Evaluación de la capacidad de los complejos $(ManS)_3$-βCD:chaperona farmacológica de aumentar la actividad de la β-glucocerebrosidasa en fibroblastos de pacientes de Gaucher.

La actividad chaperona de los sp^2-iminoazúcares **6S-NOI-NJ** y **6S-NAdB-NJ** y de sus complejos con **2** se ha evaluado frente a tres fibroblastos mutantes GD, F213I/F213I (dominio catalítico, neuronopático), N370S/N370S (dominio catalítico, no-neuronopático) y L444P/L444P (domino no-catalítico, neuronopático) y en fibroblastos nativos sanos. Las células se cultivaron en ausencia y en presencia de cantidades crecientes de PCs (3 y 30 μM) y de los correspondientes complejos 1:1 **2**. Tras cuatro

días, la actividad GCasa se determinó mediante medidas fluorimétricas usando el 4-metilumbeliferil α-D-glucopiranósido. Como se observa en la Figura 3.7.A, ni las chaperonas **6S-NOI-NJ** y **6S-NAdB-NJ**, ni sus complejos afectan a la actividad de la enzima no mutante, lo que indica que la función lisosomal no se ve inhibida ni a la concentración más alta (30 μM). Las dos PCs **6S-NOI-NJ** y **6S-NAdB-NJ** aumentaron la actividad de la GCasa en fibroblastos F213I/F213I hasta 3 veces, de manera dosis-dependiente (Figura 3.7.B). La mutación N370S/N370S, no-neuronopática, fue mucho menos sensible a las PCs; únicamente la **6S-NAdB-NJ** a concentración 3 μM indujo un aumento de actividad (2-veces; Figura 3.7.C). No se observó prácticamente ningún efecto en el caso de la mutación L444P/L444P, Figura 3.7.D, de acuerdo con los resultados previamente descritos para esta familia de PCs.[15a] En cualquier caso, el comportamiento de las dos PCs es independiente de la complejación con **2**, lo que confirma que existe una transferencia rápida de la PC desde la cavidad de la βCD al sitio activo de la GCasa.



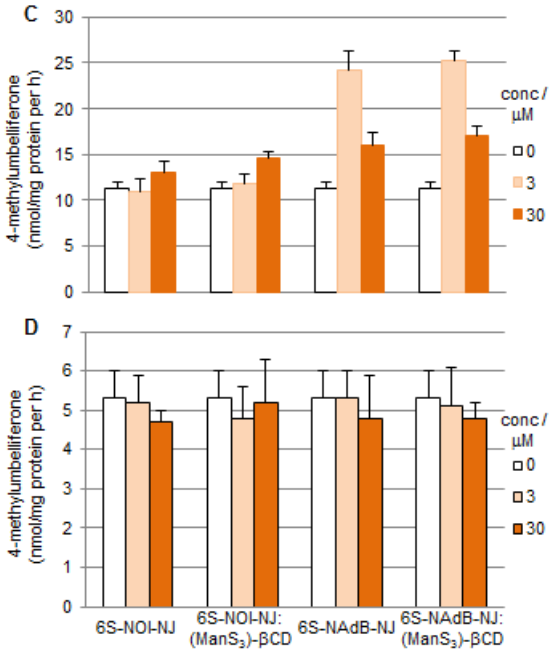


Figura 3.7. Actividad chaperona de **6S-NOI-NJ** y **6S-NAdB-NJ** y de los correspondientes complejos 1:1 con **2** en fibroblastos control (A) y mutantes (B), F213I/F213I; C, N370S/N370S; D, L444P/L444. Las células se cultivaron durante cuatro días en presencia y ausencia de las chaperonas. La actividad de la GCasa lisosomal se midió mediante la producción de 4-metilumbeliferona.

3.2.6. Reconocimiento de los complejos (ManS)$_3$-βCD:chaperona farmacológica por macrófagos.

Para confirmar el potencial del transportador **2** para unirse al MMR de los macrófagos, se ha llevado a cabo un estudio in vitro de la capacidad de adhesión a macrófagos peritoneales de ratón. La capacidad de adhesión se ha determinado usando un procedimiento adaptado al descrito por Muller y Schuber[29] que se basa en el aumento de fluorescencia del ácido 6-*p*-toluidin-2-nafthalenosulfónico (TNS)

cuando se incluye en la cavidad hidrófoba de la βCD y de los derivados de βCD para formar complejos 1:1.[1] La células se incubaron con concentraciones crecientes del complejo 1:1 TNS:**2** a 4 ºC para evitar la fagocitosis y la cantidad de complejo asociado a la membrana de la célula se determinó mediante fluorimetría. Como se muestra en la Figura 3.8.A, los niveles de fluorescencia se incrementaron de manera dependiente de la dosis tras incubación con el complejo TNS:**2**, mientras que el complejo TNS:βCD, usado como control, mostró valores de fluorescencia mínimos. Es interesante señalar que la adhesión de TNS:**2** a los macrófagos se inhibió en presencia de cantidades crecientes de **2** o de los complejos 1:1 **2**:**6S-NOI-NJ** y **2**:**6S-NAdB-NJ** (Figura 3.8.B, IC_{50} 42, 39 y 36 μM, respectivamente). Estos resultados apoyan el hecho de que el transportador y los correspondientes complejos con la PC o TNS compiten por el mismo receptor de la superficie de los macrófagos de acuerdo con la participación del MMR en el proceso de adhesión.

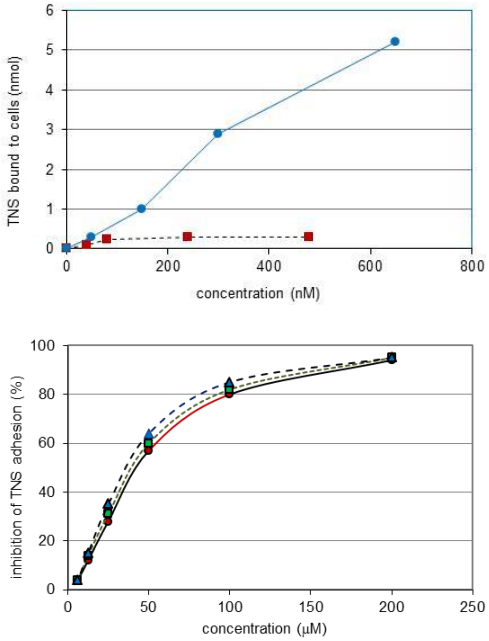
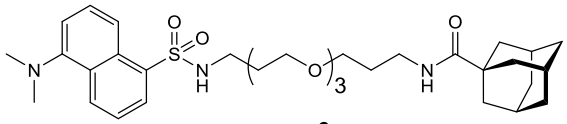


Figura 3.8. (A) Adhesión de los complejos 1:1 TNS:$(ManS)_3$-βCD determinada mediante fluorimetría (línea ●) y TNS:βCD (línea de trazos ■) a la membrana celular de macrófagos de ratón y (B) ensayo de desplazamiento competitivo del TNS TNS usando **2** (línea ○), y los complejos 1:1 **6S-NOI-NJ**:$(ManS)_3$-βCD (línea □) y **6S-NAdB-NJ**:**2**(línea △).

En trabajos anteriores hemos descrito el empleo de radicales dansilo para estudiar la internalización de iminoazúcares sp^2 en fibroblastos.[9] Sin embargo, la presencia de una sonda fluorescente relativamente voluminosa puede alterar la capacidad de atravesar membranas biológicas y las propiedades de difusión de la chaperona, en comparación con las de la molécula sin etiquetar lo que limita el alcance de las conclusiones. Con objeto de confirmar si los complejos PC:**2** se internalizan en macrófagos a través de receptores MMR, hemos diseñado un tipo de ensayos competitivos que utilizan la sonda fluorescente dansilo-adamantano **6** y el manosil derivado **3** (Figura 3.9.) como modelos del receptor βCD y del ligando MMR, respectivamente. Los experimentos se han llevado a cabo en macrófagos diferenciados desde monocitos humanos THP-1, usando microscopia de fluorescencia 3D. Este tipo de células presentan muchas propiedades análogas a las de los macrófagos humanos primarios, incluyendo la expresión de macrófagos MMR.



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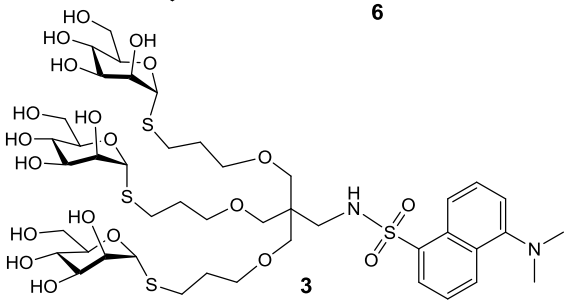

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Figura 3.9. Estructuras de las sondas dansiladas **6** y **3** empleadas para la monitorización mediante microscopía confocal de la internalización de los complejos PC:CD.

En experimentos de control se observó que la incubación de las células con el compuesto **3** (400 μM) da lugar a una fluorescencia verde intensa en el citoplasma que resulta abolida al añadir un exceso de levadura de manano de acuerdo con una internalización del conjugado a través del receptor MMR. Resultados análogos se obtuvieron al sustituir **3** por el complejo 1:1 **2**:**6** a la misma concentración (Figura 3.10.A), pero no cuando se incuban las células con el compuesto **6** sin complejar, lo que indica que el transportador **2** promueve la internalización del huésped después de ser reconocido por el receptor MMR. En experimentos en paralelo se comprobó que un exceso del sp^2-iminoazúcar **6S-NAdB-NJ** no afecta la internalización de **3**, descartando una interacción directa de la chaperona con el receptor MMR. Sin embargo, en el caso del complejo 1:1 **2**:**7** la fluorescencia disminuyó considerablemente tras añadir cantidades crecientes de **6S-NAdB-NJ** antes de la incubación (Figura 3.10.B y C). Este resultado es compatible con el desplazamiento parcial de la sonda fluorescente **6** por la chaperona farmacológica y la internalización posterior del complejo **2**:**6S-NAdB-NJ** no fluorescente a través de la ruta mediada por el receptor MMR. En la Figura 3.10.D se muestra una representación cuantitativa de las intensidades relativas de fluorescencia intracelular. Aunque con las limitaciones intrínsecas de cualquier método indirecto, el conjunto de resultados apoya la hipótesis de que el derivado **2** interacciona específicamente con el receptor MMR a través de la antena trimanosilada y que esta interacción desencadena la internalización del correspondiente complejo de inclusión con la chaperona en el interior de los macrófagos.

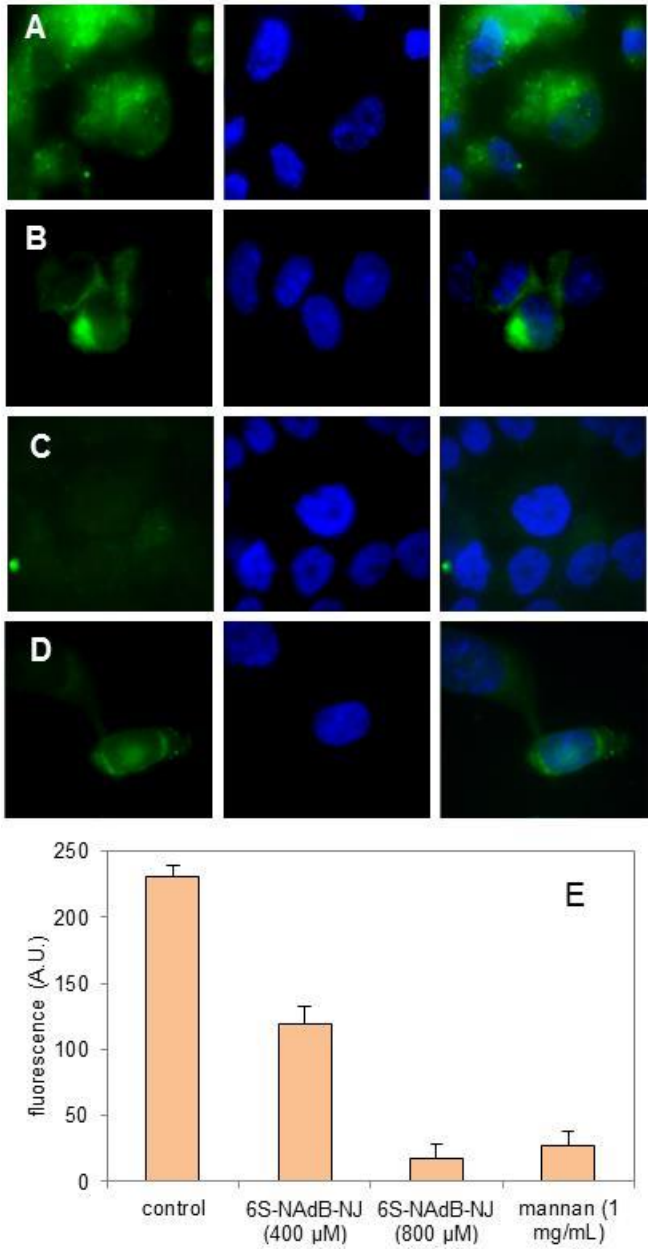

Figura 3.10. Imágenes de microscopía de fluorescencia de células monocíticas humanas THP-1 diferenciadas a macrófagos incubadas con el complejo **2:6** (400 μM) (fila A) y en presencia de **6S-NAdB-NJ** (400 o 800 μM, filas B y C, respectivamente) o manano de levadura (1 $mg \cdot mL^{-1}$, fila

D). La Figura E muestra la fluorescencia intracellular después de tratamiento con el complejo **2**:**7** (400 μM) solo (control) y en presencia de **6S-NAdB-NJ** (400 o 800 μM) o manano de levadura (1 $mg \cdot mL^{-1}$).

Estos resultados suponen una prueba de concepto acerca de la idoneidad del derivado manosilado **2** como un sistema de transporte eficaz a macrófagos de chaperonas farmacológicas, con estructura de sp^2-iminoazúcares anfífilicas, para la enfermedad de Gaucher. La estructura del transportador monosustituido y multivalente ha sido diseñada para favorecer las propiedades de inclusión de los sustituyentes hidrofóbicos de los candidatos **6S-NOI-NJ** y **6S-NAdB-NJ** al tiempo que se garantiza la capacidad de reconocimiento por lectinas específicas de manosa. Los dos objetivos se han demostrado en primer lugar usando β-glucosidasas y lectina Con A comerciales y se han validado posteriormente con β-glucocerebrosidasa humana y lectina MMR humana (recombinante). La complejación de las chaperonas con **2** preserva su actividad, los correspondientes complejos reconocen específicamente a los receptores MMR de la superficie de los macrófagos, el tipo de célula más afectado en pacientes de GD, y este fenómeno permite la internalización en el macrófago.

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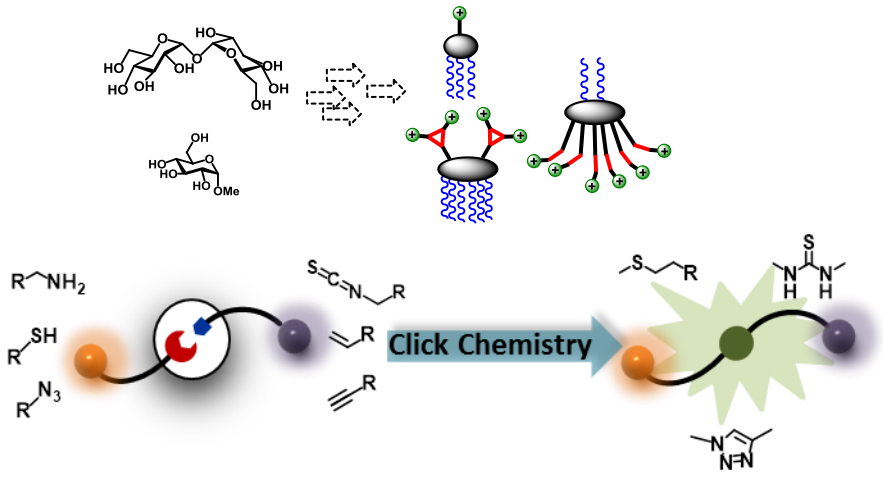
Capítulo 4

Síntesis y caracterización de derivados catiónicos anfifílicos de glucosa y trehalosa.

Abstract: Carbohydrate-based surfactants are an important class of amphiphilic. They are usually non toxic and non immunogenic, thus being safer than conventional ionic surfactants. In the last years there has been an increasing interest in using these novel systems as liquid crystals, drug candidates for a wide range of diseases, or as delivery systems for gene therapy. In this chapter, we synthesized a new class of trehalose and glucose based cationic glycoamphiphiles by synthetic strategies based on ‘’click chemistry’’ reactions such as azide-alkine cycloaddition, thiol-ene coupling or thiourea forming reactions, in order to easily access to molecular diversity with good to excelents yields. Furthermore, molecular properties of aggregation and self assembling capabilities were measured in aqueous media.



4.- Síntesis y caracterización de derivados catiónicos anfifílicos de glucosa y trehalosa.

4.1. Introducción

Los surfactantes derivados de carbohidratos constituyen una clase importante de moléculas anfifílicas.[1] El interés está principalmente asociado a su origen natural, su capacidad de detergencia y su baja toxicidad, lo que los hace a priori más ecológicos que los surfactantes iónicos o no iónicos convencionales.[2] Entre sus aplicaciones más extendidas está sus usos como agentes emulsificantes[3], como agentes solubilizadores de proteínas[4] y, en relación directa con esta Tesis, como sistemas de transporte de fármacos[5] o material génico.[6]

Entre los sistemas artificiales de transporte de genes y fármacos basados en lípidos catiónicos, en los últimos años han despertado un interés creciente los llamados surfactantes gemelos[7]. ''Surfactante gemelo''[8] o ''gemini'' es el nombre asignado a una familia de moléculas anfifílicas que poseen, en secuencia, una larga cadena hidrocarbonada, un grupo iónico, un espaciador, un segundo grupo iónico, y otra cola hidrocarbonada, o dicho de otra manera, estarían constituidos por dos surfactantes idénticos, unidos por un espaciador (Figura 4.1.) presentando por tanto 2n cadenas lipófilas y 2m cabezas polares (siendo *n* el número de cadenas lipófilas por unidad de surfactante y *m* los grupos polares por unidad) que pueden ser positivas, negativas, o neutras. El segmento conector tiene naturaleza variable pudiendo ser corto, rígido, flexible, polar o apolar. Esta peculiaridad estructural les confiere propiedades físico-químicas diferentes a lo que sería esperable, si los comparamos con los correspondientes monómeros. En general, un surfactante gemelo tendrá una menor Concentración Micelar Crítica (CMC, concentración de surfactante a partir de la cual se forman micelas espontáneamente) que su análogo monovalente. Como sistemas de transporte de fármacos y material génico[9] aportan además una disminución de la concentración necesaria para aplicaciones *in vivo* así como menor toxicidad.[10]

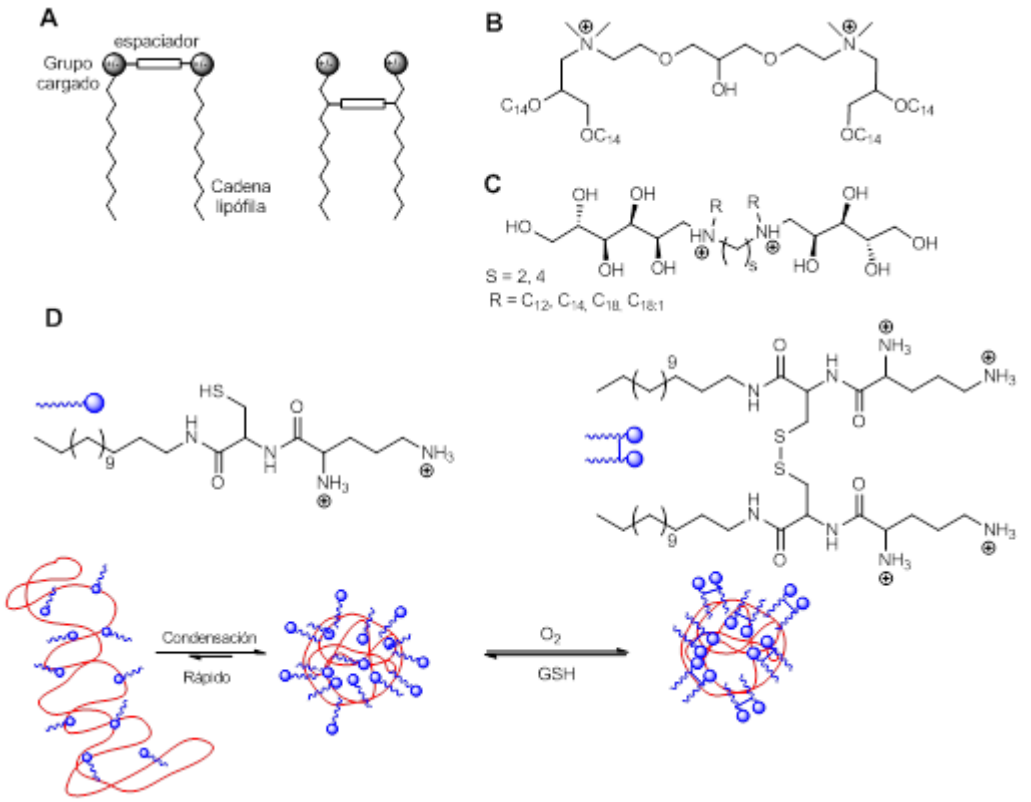


Figura 4.1. (a) Representación esquemática de los posibles tipos de surfactantes gemelos; (b) surfactante gemelo basado en cardiolipina; (c) surfactante gemelo que incorpora carbohidratos; (d) surfactante gemelo bioreducible descrito por Zuber y representación esquemática del proceso de oxidación-reducción de los correspondientes lipoplejos.

Dentro del marco de esta Tesis, se han desarrollado varias rutas sintéticas con objeto de acceder a estructuras monodispersas de naturaleza catiónica y anfifílica, basadas en D-glucosa y α,α'-trehalosa. En la molécula de trehalosa los dos fragmentos de glucosa son equivalentes, dada la existencia de un eje de simetría C_2, de manera que los derivados anfifílicos correspondientes pueden considerarse como surfactantes gemelos constituidos por dos subunidades de monosacárido idénticas unidas por un átomo de oxígeno como espaciador. Además, la confluencia de dos efectos anoméricos y exoanoméricos en la unión interglicosídica proporciona a la α,α'-trehalosa una estructura rígida con una diferenciación facial marcada, estando los hidroxilos primarios orientados en sentido opuesto a los hidroxilos secundarios. Los derivados anfifílicos de trehalosa pueden considerarse por tanto al mismo tiempo anififílicos faciales no macrocíclicos, lo que podría proporcionarles propiedades favorables de autoorganización y capacidad para atravesar membranas biológicas. En este sentido, un estudio comparativo entre derivados anfifílicos de la D-glucosa y la α,α'-trehalosa resulta especialmente interesante. Hemos considerado para ello dos posibles arquitecturas, en función de la presentación de las cabezas catiónicas y las cadenas lipófilas y que, por analogía con la

nomenclatura comúnmente utilizada para los derivados anfifílico de ciclodextrinas, hemos deominado como sistemas tipo ‘’falda’’ y tipo ‘’medusa’’. Los derivados del primer tipo presentan los grupos catiónicos en las posiciones primarias de los carbohidratos y los grupos lipídicos en las secundaria. En los sistemas tipo ‘’medusa’’, también llamados ‘’reversos’’, la disposición de los dominios catiónico y lipófilo es la opuesta. En ambos casos se han utilizado para las etapas de multiconjugación las reacciones tipo “click” de acoplamiento azida-alquino catalizada por cobre(I) (CuAAC) y el acoplamiento amina-isotiocianato para dar tioureas.

4.2. Resultados y Discusión

4.2.1. Sistemas tipo “falda”.

Para la preparación de una primera serie de derivados policatiónicos anfifílicos de tipo ‘’falda’’ derivados del metil α-D-glucopiranósido (**11**, **47**, **50**) y de la α,α’-trehalosa (**70**, **101**, **103**) (Figura 4.2.), hemos partido de los correspondientes 6-desoxi-6-yodo y 6-6’-didesoxi-6,6’-diyodo derivados y se ha seguido la secuencia de reacciones que se presenta en el Esquema 4.1.[11,12] En primer lugar, la peracilación de la cara secundaria de los yododerivados con anhídrido hexanoico y 4-(*N*,*N*-dimetilamino)piridina (DMAP) condujo a los derivados **7** y **67**. A continuación, la sustitución nucleófila de los átomos de yodo con 2-(*terc*-butoxicarbonilamino)etanotiol (cisteamina *N*-Boc), en DMF y en presencia de $CsCO_3$ (→ **10** y **69**), seguido de desprotección de los grupos carbamato con TFA y posterior liofilización en presencia de HCl diluido, permitió aislar las aminas **11** y **70** en forma de hidrocloruros con buenos rendimientos globales (58-85%) (Esquemas 4.1 y 4.2.). Trabajos anteriores indican que la presencia del espaciador de etiltio incrementa notablemente la nucleofilia de los grupos amino en comparación con derivados en los que el grupo amino se encuentra directamente sobre la posición C-6.[13] En nuestro caso, los compuestos **11** y **70** nos han permitido abordar con eficacia la preparación de una serie de tioureas policatiónicas mediante acoplamiento nucleofílico con los isotiocianatos. Así, la reacción con 2-[bis[2-(*terc*-butoxicarbonlamino)etil]amino]etil isotiocianato[14] **167** y 2-*terc*-butoxicarbonilamino)etil isotiocianato[15] **168**, en diclorometano condujo s los aductos **45**, **49**, **100** y **102** que, tras desprotección de los grupos carbamato condujo a los aminotioureido derivados **47**, **50**, **101** y **103** con rendimientos excelentes (Esquemas 4.1 y 4.2).

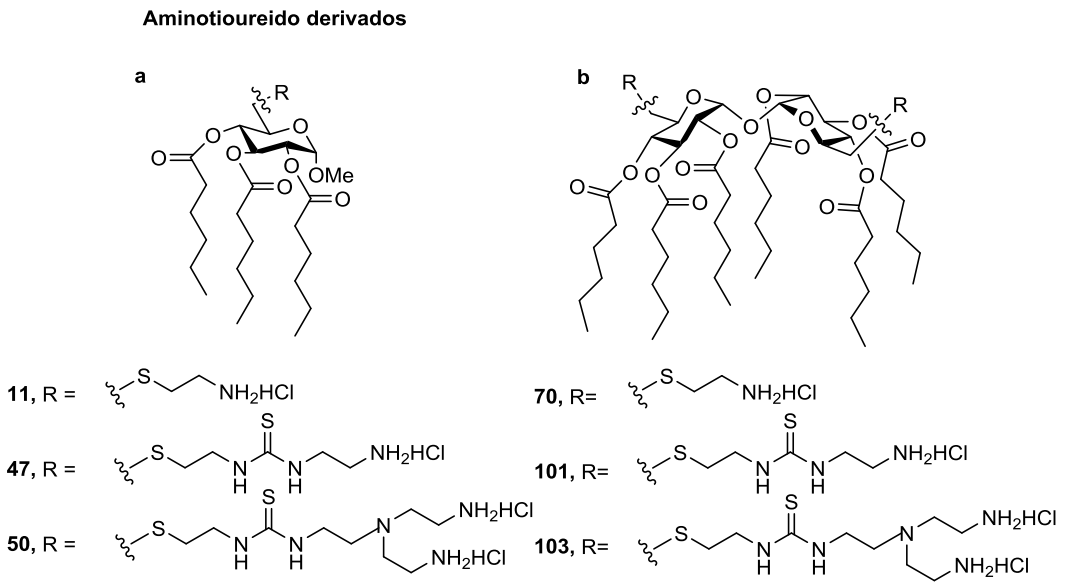
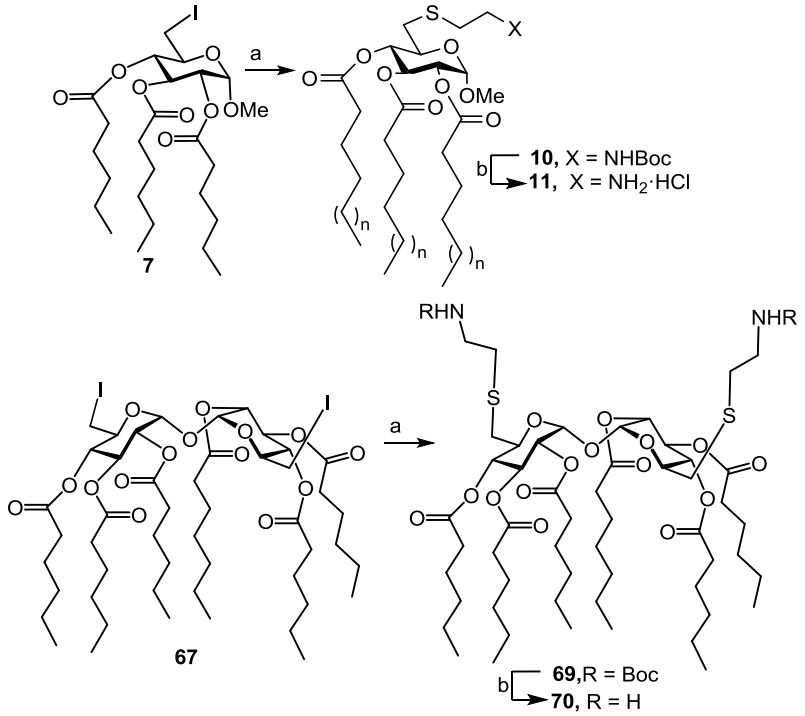
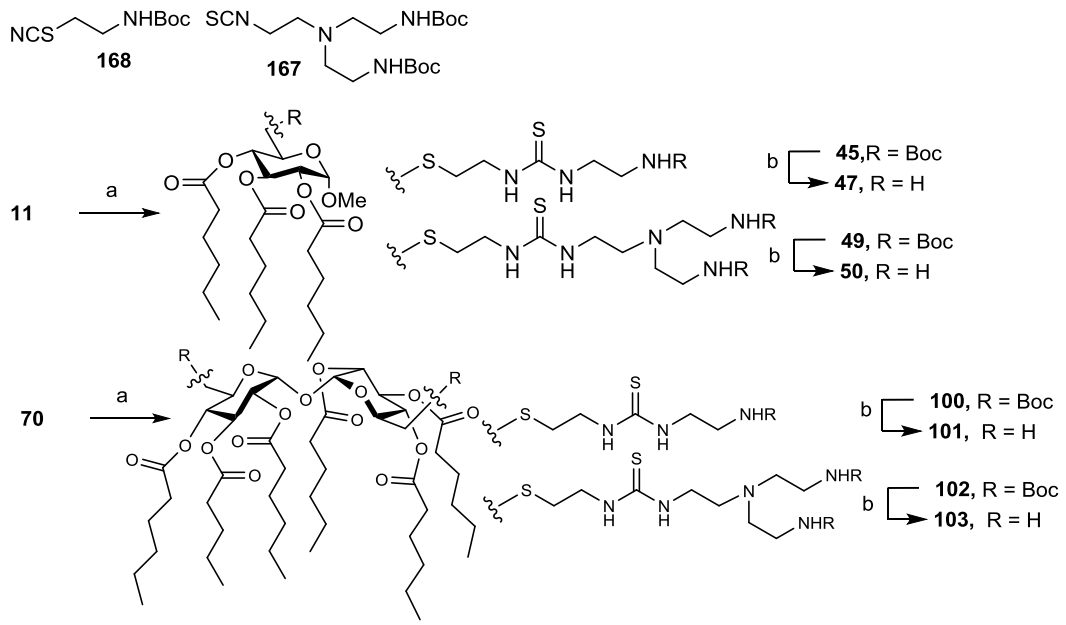


Figura 4.2. Estructura de los derivados policatiónicos anfifílicos de glucosa y trehalosa de tipo falda funcionalizados con grupos aminotioureido.



Esquema 4.1. Síntesis de los derivados policatiónicos anfifílicos de glucosa y trehalosa **11** y **70**. Reactivos y condiciones: (a) $BocHN(CH_2)_2SH$, Cs_2CO_3, DMF, Ar, 60 ºC, 48 h, (58 y 85% para **10** y **69**); (b) TFA-DCM, 1 h, H^+, cuantitativo.



Esquema 4.2. Síntesis de las tioureas policatiónicas anfifílicas de glucosa y trehalosa tipo "falda" **47**, **50**, **101 y 103**. Reactivos y condiciones: (a) **167**/**168**, Et_3N, DCM, Ar, 16, 42-73%; (b) TFA-DCM, t.a., 1 h, cuantitativo.

La pureza y homogeneidad de las tioureas policatiónicas anfífilicas **47**, **50**, **101 y 103** se confirmó mediante espectrometría de masas (ESI-MS), análisis elemental y espectroscopías de ^{1}H y ^{13}C RMN (Figura 4.3.). Los espectros registrados a temperatura ambiente mostraron el ensanchamiento característico de las señales debido a la rotación lenta alrededor de los enlaces C−N de los grupos carbamato y tiourea. Es interesante destacar que los espectros de ^{1}H y ^{13}C RMN de los derivados de trehalosa **101** y **103** muestran un único sistema de spin, confirmando la simetría C_2 de estos derivados (Figura 4.4.).

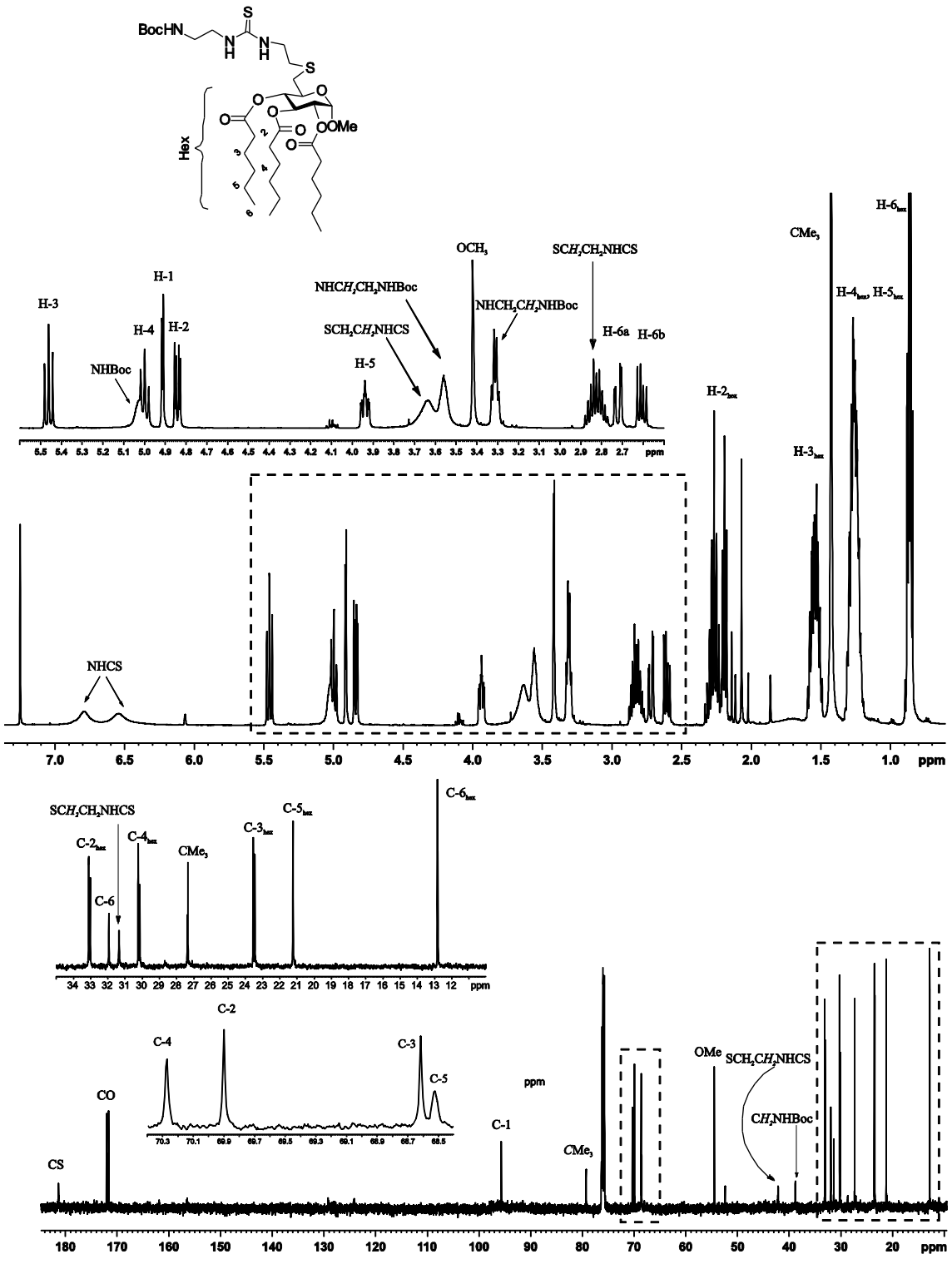


Figura 4.3. Espectros de ^{1}H y ^{13}C RMN (300 MHz, 75.5 MHz, $CDCl_3$) de **45.**

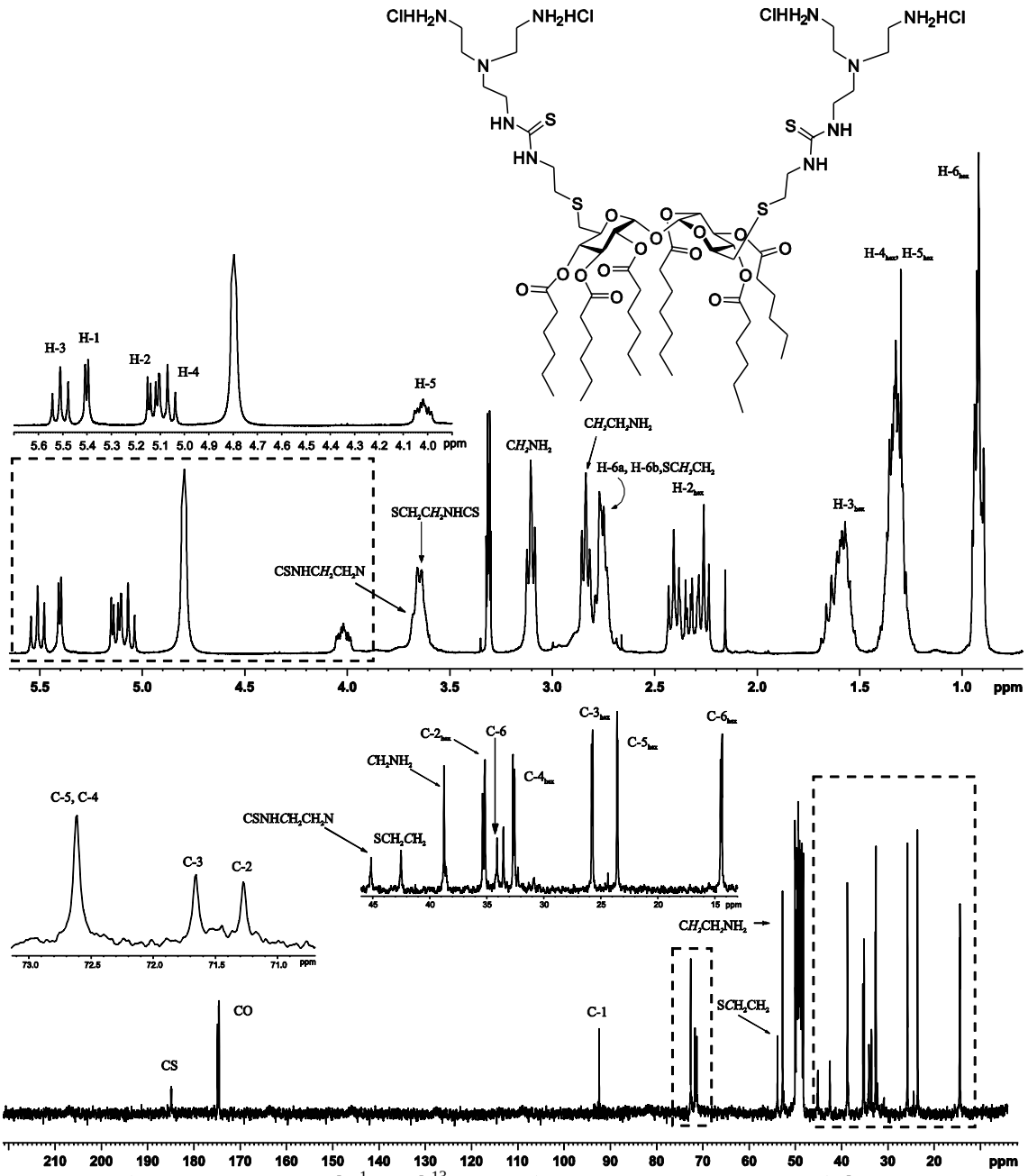


Figura 4.4. Espectros de ^{1}H and ^{13}C RMN (300 MHz, 75.5 MHz, CD_3OD) de **103**.

Con objeto de disponer de un conjunto amplio de compuestos para estudios de estructura-propiedades de autoasociació-actividad, hemos preparado una serie adicional de derivados policatiónicos anfifílicos empleando la reacción de CuAAC. La incorporación del anillo de triazol confiere mayor rigidez a los sistemas, lo que debe influir en sus propiedades conformacionales y afectar a la estabilidad de los posibles agregados. Además, en lo referente a su aplicación como vectores de genes, las propiedades de liberación del material génico en el endosoma, a pH ácido, también pueden verse afectadas debido a las características ácido-base del anillo heterocíclico (pK_a del anillo de triazol = 6). Las reacciones de acoplamiento de tipo CuAAC entre los 6-azido-6-desoxi derivados de glucosa y trehalosa **9** y **68** y los derivados

propargilados bis[2-*terc*-butoxicarbonilamino)etil]propargilamina (**169)** y *N*-(*terc*-butoxicarbonil)propargilamina (**170)** transcurrieron con rendimientos superiores al 85% en una mezcla de ^{t}BuOH-H_2O 1:9 empleando un catalizador de Cu(I) soportado sobre sílica, **Si-BPA·Cu**$^{+16}$ (→ **51**, **53**, **104** y **106**; Figura 4.5.). La hidrólisis ácida posterior de los grupos carbamato condujo a los conjugados **52, 54, 105** y **107** en forma de los correspondientes hidrocloruros tras liofilización de una disolución diluida de HCl (Esquema 4.3).

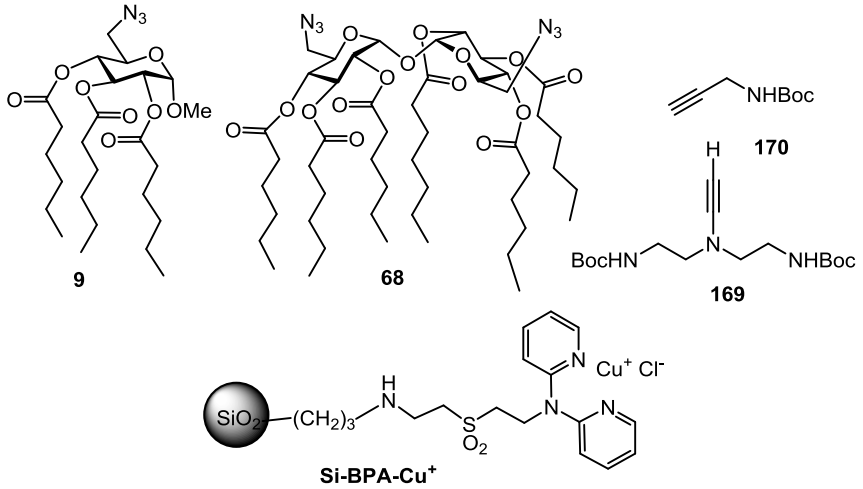
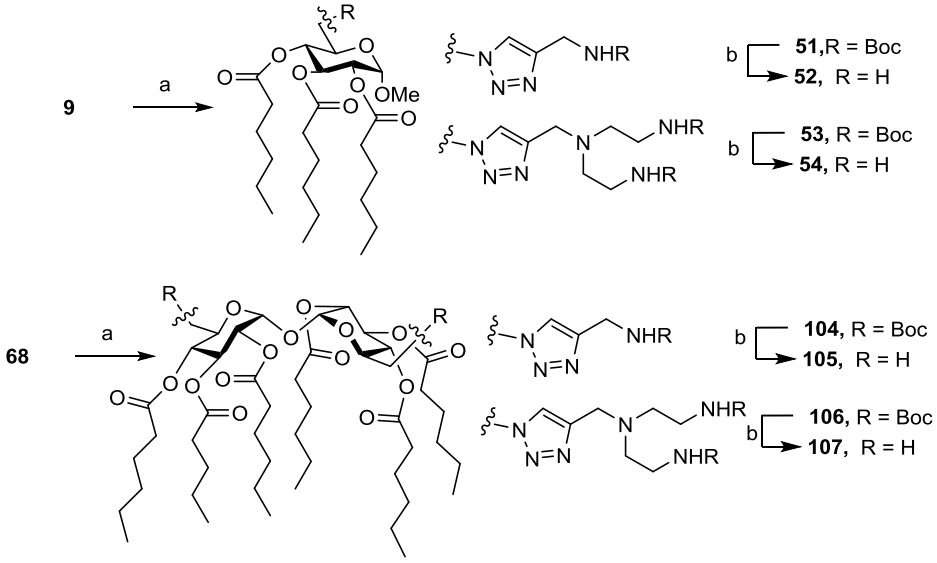
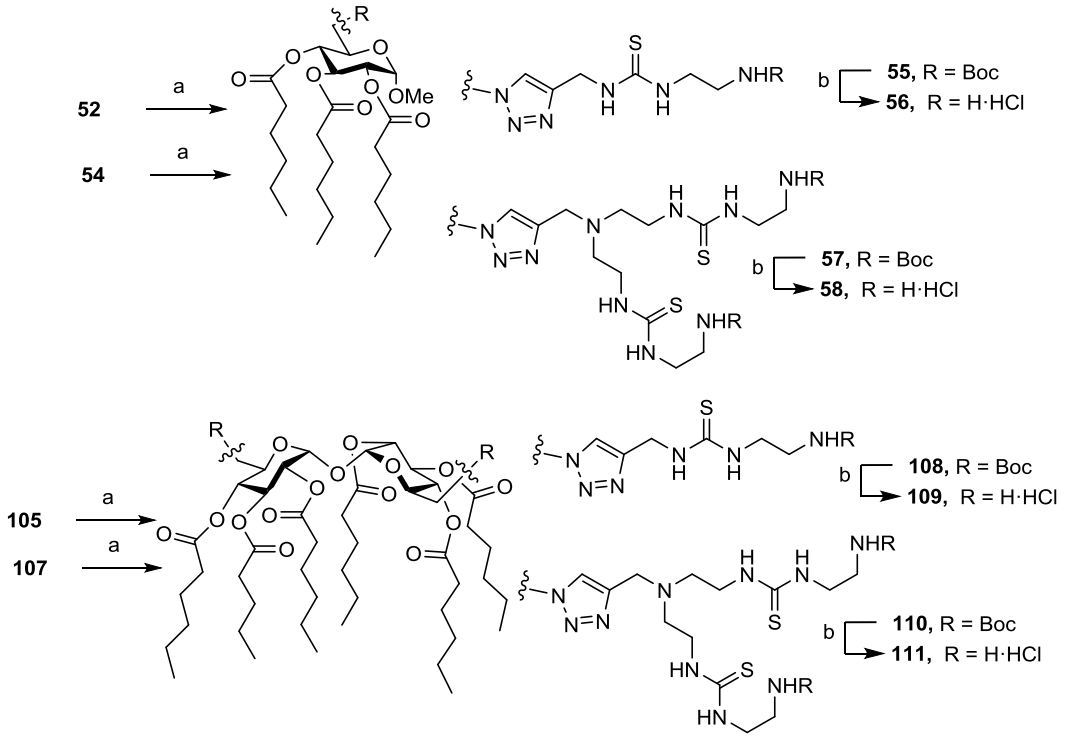


Figura 4.5. Estructura de las azidas derivadas de glucosa y trehalosa (**9, 68),** los alquinos *N*-Boc protegidos (**169**, **170**) y el catalizador de Cu(I) soportado sobre sílica empleados para la preparación de los ‘’click’’ derivados catiónicos anfifílicos de glucosa y trehalosa.



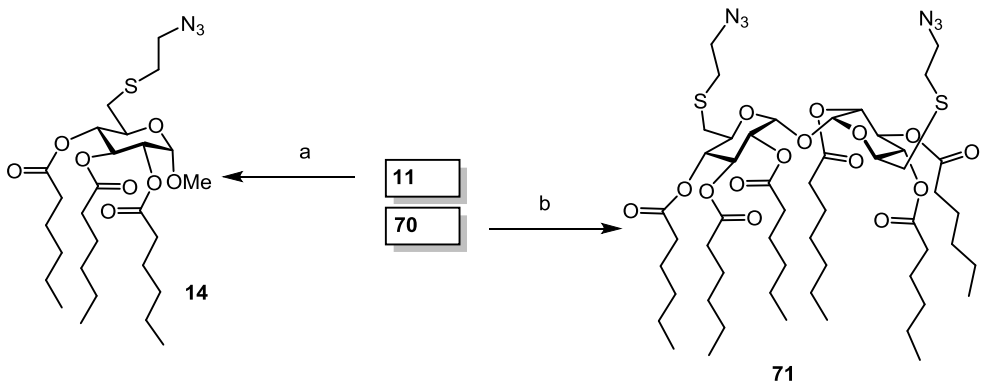
Esquema 4.3. Síntesis de los derivados policatiónicos anfifílicos de glucosa y trehalosa tipo “falda” **52**, **54**, **105 y 107**. Reactivos y condiciones: (a), **Si-BPA·Cu**$^{+}$**,** 9:1 H_2O-^{t}BuOH, 85 ºC, 36 h, 78%-cuantitativo. (b)TFA-DCM, t.a., 1 h. H^{+}, 96%-cuantitativo.

A partir de las aminas triazólicas **52**, **54**, **105** y **107** se prepararon además los compuestos **56**, **58**, **109** y **111**, por condensación con el isotiocianato **168** (rendimiento 52-69%; Esquema 4.4). Tras purificación de los aductos **55**, **57**, **108** y **110** por cromatografía en gel de sílice seguida de hidrólisis ácida de los grupos carbamato y liofilización a partir de una disolución de HCl diluido, se aislaron los derivados policatiónicos objetivo en forma de hidrocloruros con excelentes rendimientos.



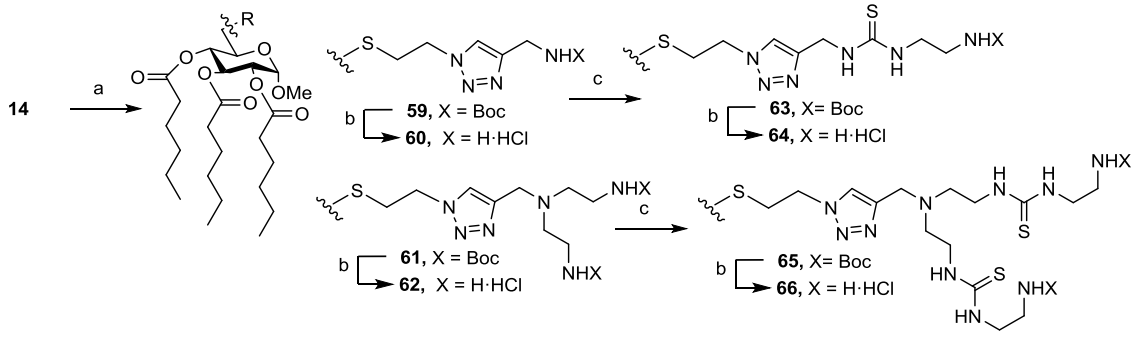
Esquema 4.4. Síntesis de los derivados policatiónicos anfifílicos de glucosa y trehalosa tipo "falda" **56**, **58**, **109**, y **111**. Reactivos y condiciones: (a) **168**, Et_3N, DCM, Ar, 16 h, 52-69%; (b), TFA-DCM, t.a., 1 h. H^+, 91%-cuantitativo.

Una estrategia adicional para incrementar la variabilidad estructural en la serie de derivados triazólicos policatiónicos ha consistido en la introducción de un espaciador de cisteamina. Para ello preparamos en primer lugar las cisteaminilazidas derivadas de glucosa y trehalosa **14** y **71** a partir de los cisteaminil derivados **11** y **70**, haciendo uso de una reacción de transferencia de grupo diazo con triflil azida (TfN_3) y $CuSO_4 \cdot 10\ H_2O$ como catalizador. En nuestro caso la TfN_3 se preparó in situ a partir del anhídrido trifluorometanosulfónico, mediante una variación del procedimiento descrito por Wong,[17] que utiliza como disolvente una mezcla de tolueno y agua. La adición a la amina de Cu(II), como catalizador, y de la solución de TfN_3 permite aislar, tras 24 horas a temperatura ambiente, los azidocisteaminil derivados **14** y **71** con rendimientos de buenos a excelentes (93% y 59%, respectivamente; Esquema 4.5).

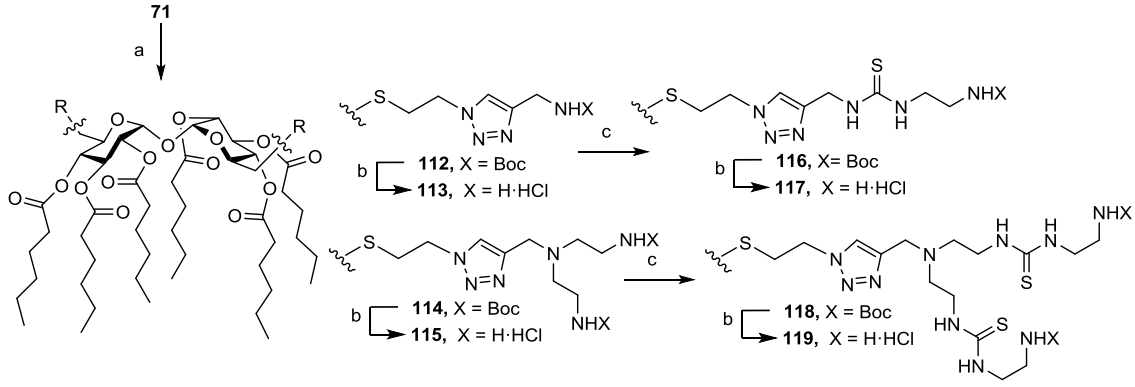


Esquema 4.5. Síntesis de los azidocisteaminilderivados de glucosa y trehalosa **14** y **71**. Reactivos y condiciones: (a), TfN_3, $CuSO_4 \cdot 5\ H_2O$ MeOH, t.a. 24 h, (93% y 59% para **14** y **71**,).

El acoplamiento click de tipo CuAAC de las azidas **14** y **71** y los propargilderivados **169** y **170** en las condiciones de catálisis heterogénea anteriormente indicadas, condujo a los conjugados **59**, **61**, **112** y **114** que, tras hidrólisis en condiciones ácidas (→**60**, **62**, **113**, **115**), posterior acoplamiento con el 2-aminoetilisotiocianato *N*-Boc protegido (→**63, 65, 116** y **118**), tratamiento con TFA y liofilización de una disolución de HCl diluido, condujo, con excelentes rendimientos, a los derivados objetivo **64**, **66**, **117**, **119** (Esquemas 4.6. y 4.7.).



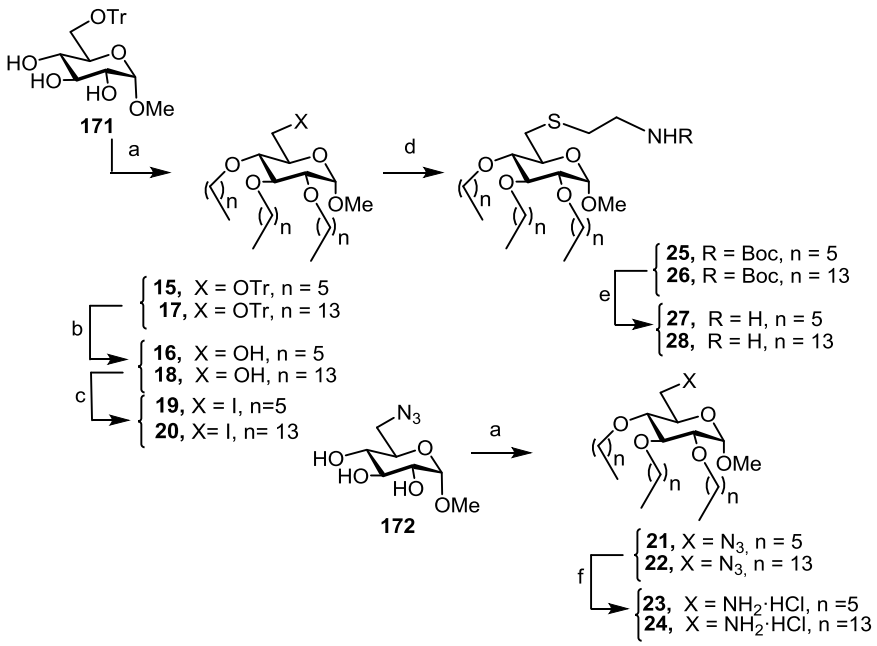
Esquema 4.6. Síntesis de los derivados policatiónicos anfifílicos de glucosa **60**, **64**, **62** y **66**. Reactivos y condiciones: (a) **169** o **170**, Si-BPA·Cu^+, 9:1 H_2O-^{t}BuOH, 85 ºC, 24 h, 87-95%; (b) TFA-DCM, t.a., 1 h. H^+, 90%-cuantitativo; (c), Et_3N, DCM, Ar, 48 h, 65-90%.



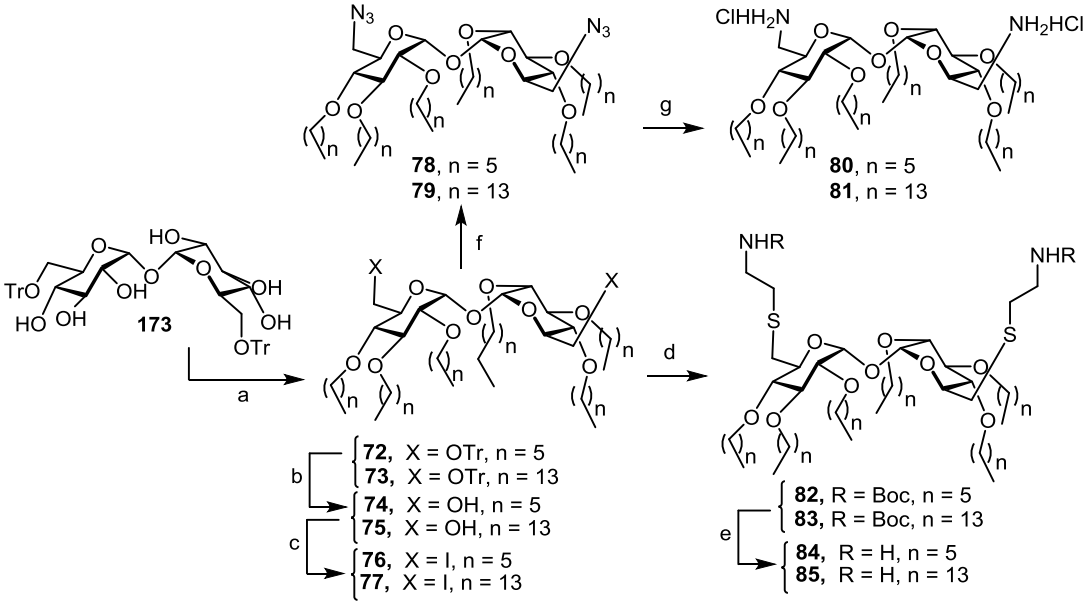
Esquema 4.7. Síntesis de los derivados policatiónicos anfifílicos de trehalosa **113**, **117**, **115** y **119**. Reactivos y condiciones: (a) **169** o **170**, **Si-BPA·Cu$^+$,** 9:1 H_2O-^{t}BuOH, 85 ºC, 24 h, 91%-cuantitativo; (b), TFA-DCM, t.a., 1 h., 90%-cuantitativo; (c) Et_3N, DCM, Ar, 48 h, 54-80%.

La colección de compuestos de tipo "falda" se amplió con una serie de derivados funcionalizados con grupos alquilo de diferente longitud , en lugar de ésteres, en los hidroxilos secundarios. La síntesis de los 6-amino-6-desoxi derivados de glucosa trialquilados **23** y **24** (Esquema 4.9.) se llevó a cabo mediante una secuencia sintética en dos etapas que implica la alquilación del metil 6-azido-6-desoxiglucopiranósido **172**[18] (→**21**, **22**) y la reducción por hidrogenación catalítica o por reacción de Staüdinger con TPP seguida de hidrólisis básica con hidróxido amónico del correspondiente fosfaceno intermedio.[19] Los aminoglicolípidos finales **23** y **24** se aislaron como hidrocloruros.

Para la preparación de los derivados que incorporan el grupo cisteamina hemos partido del metil 6-*O*-tritil-α-D-glucopiranósido **171** (Esquema 4.8.) y de la 6,6'-di-*O*-tritil-α,α'-trehalosa **173** (Esquema 4.9), que se sometieron a alquilación exhaustiva con el correspondiente bromoalcano en presencia de NaH (→**15**, **17**, **72**, **73**) seguida de hidrólisis ácida del grupo tritilo con BF_3-Et_2O (→**16**, **18**) ó PTSA (→**74**, **75**) aislándose los derivados con el grupo hidroxilo OH-6 libre con rendimientos del 71-75%. La yodación directa del hidroxilo primario con el sistema TPP-imidazol-yodo (método de Garegg[20]) condujo a los 6-yodoazúcares **19, 20**, **76** y **77** con rendimientos excelentes, que tras sustitución nucleofílica con el derivado *N*-Boc protegido de cisteamina, hidrólisis en condiciones ácidas (→**25**, **26**, **82**, **83**), tratamiento con HCl diluido y posterior liofilización proporcionaron los derivados desprotegidos **27**, **28**, **84** y **85** en forma de hidrocloruros, con buenos rendimientos globales. En el caso de la trehalosa, los 6,6'-diamino derivados **80** y **81** se obtuvieron por sustitución con NaN_3 de los yododerivados **76** y **77** y posterior reducción de las azidas con TTP y NH_4OH (→**80**, **81**) seguido de liofilización en presencia de HCl.

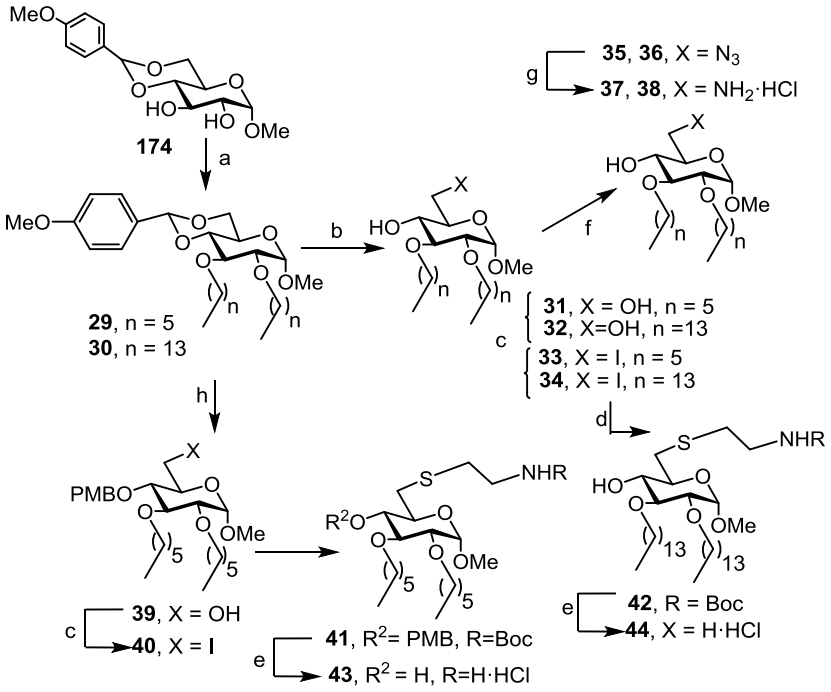


Esquema 4.8. Síntesis de los derivados policatiónicos alquilados de glucosa **23**, **24**, **27 y 28**. Reactivos y condiciones: (a) 1-Bromohexano ó 1-Bromotetradecano, NaH, DMF, t.a.→60 ºC, 16 h, 48-96%; (b) $BF_3{\cdot}Et_2O$, MeOH, t.a., 2 h, 47-75%; (c) I_2, TPP, imidazol, tolueno, 70 ºC, 5 h, 89-91%; (d) $HS(CH_2)_2NHBoc$, Cs_2CO_3, DMF, 60 ºC, 16 h, 70-71%; (e) 1:1 TFA-DCM, t.a., 15 min, 97-99% (f) H_2, Pd/C, 2 h, 87% (g) TPP, THF, NH_4OH, 50 ºC, 16 h, 82%.



Esquema 4.9. Síntesis de los derivados policatiónicos alquilados de trehalosa **81, 75, 84 y 85**. Reactivos y condiciones: (a) 1-Bromohexano ó 1-Bromotetradecano, NaH, DMF, t.a.-60 ºC, 16 h, 77-92%; (b) PTSA, DCM-MeOH, t.a., 3 h, 47-48%; (c) I_2, TPP, imidazol, tolueno, 70 ºC, 5 h, 94-96%; (d) $HS(CH_2)_2NHBoc$, Cs_2CO_3, DMF, 60 ºC, 16 h, 96-99%; (e) 1:1 TFA-DCM, t.a., 15 min, 98-100% (f) NaN_3, DMF, 80 ºC, 16 h, 87-96% (g) TPP, THF, NH_4OH, 50 ºC, 16 h, 58-59%.

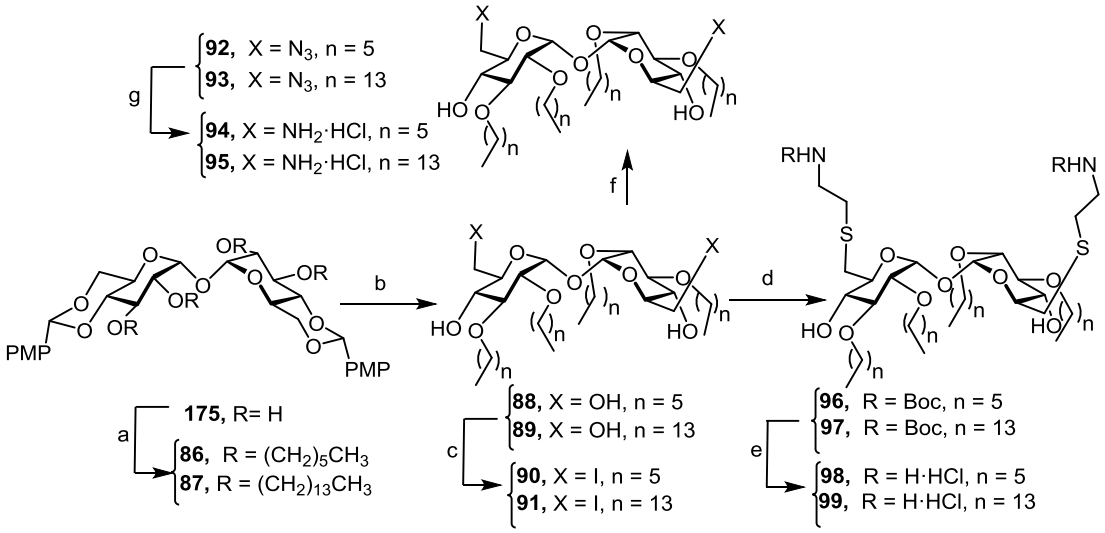
Los derivados 2,3-di-*O*-alquilados de glucosa **43** y **44** se prepararon a partir de los precursores 4,6-*O*-(*p*-metoxibencilideno) protegidos **29** y **30,**[21] obtenidos por alquilación del metil 4,6-*O*-(*p*-metoxibenciliden)-α-D-glucopiranosido **174**.[22] La apertura regioselectiva de acetal cíclico de **29** con $LiAlH_4$ condujo al éter 4-*p*-metoxibencílico **39**. Por otra parte, el acetal **30** se desprotegió completamente en las posiciones C-4 y C-6 por tratamiento con $AlCl_3$ para dar **31** y **32** con rendimientos del 87-90%. La yodación directa de los hidroxilos primarios empleando las condiciones de Garegg[11] (→**40**, **34 y 33**), seguida de desplazamiento nucleofílico de los átomos de yodo de **40** y **34** con cisteamina Boc-protegida (→**41 y 42**) y desprotección ácida del grupo carbamato condujo a **43** y **44**. Por otra parte, los 6-amino-6-desoxi derivados **37** y **38** se obtuvieron por sustitución nucleofílica de **33** y **34** con NaN_3 seguido de reducción con TPP e hidrólisis con NH_4OH, con un 54% de rendimiento (Esquema 4.10.).



Esquema 4.10. Síntesis de los derivados policatiónicos dialquilados de glucosa **43**, **44** y **34**. Reactivos y condiciones: (a) 1-bromohexano ó 1-bromotetradecano, NaH, DMF, t.a.-60 ºC, 16 h, 57%; (b) $AlCl_3$, DCM, Et_2O, 40 ºC, 4 h, 87-90%; (c) I_2, TPP, imidazol, tolueno, 70 ºC, 5 h, 60-94%; (d) $HS(CH_2)_2NHBoc$, Cs_2CO_3, DMF, 60 ºC, 16 h, 95%; (e) 1:1 TFA-DCM, t.a., 15 min, cuantitativo. (f) NaN_3, DMF, 70 ºC, Ar, 16 h, 93%; (g) TPP, THF, NH_4OH, 50 ºC, 16 h, 54%. (h) 1M $LiAlH_4$, $AlCl_3$, DCM, Et_2O, 83%.

En el caso de los derivados de trehalosa **94**, **95, 98** y **99** se siguió una estrategia análoga a la desarrollada en el caso de derivados de glucosa. El diacetal derivado de la α,α'-trehalosa **175** se sometió a alquilación de los grupos OH de las posiciones 2,2',3 y 3' con los haluros de hexilo y tetradecilo (→**86** y **87**), desprotección de los grupo acetálicos para dar los 2,3-dialquil derivados (**88** y **89**) y yodación bajo condiciones de Garegg (→**90**, →**91**). La sustitución nucleófila con

cisteamina Boc (→**96**, **97)** y la hidrólisis ácida de los grupos carbamato, permitió aislar los aminoglicolípidos **98** y **99** como hidrocloruros tras liofilizar con HCl. Por otra parte, los 6,6'-amino-6,6'-didesoxi derivados **94** y **95** se obtuvieron por sustitución nucleofílica de **90** y **91** con NaN_3 (→**92**, **93**) seguido de reducción con TPP e hidrólisis con NH_4OH con rendimientos del 86-100% (Esquema 4.11.).

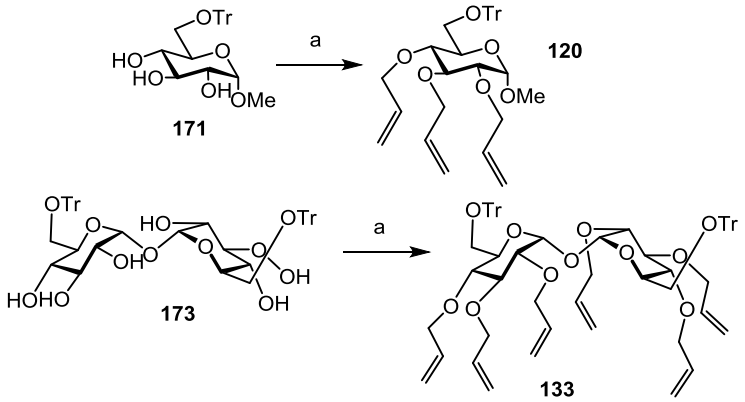


Esquema 4.11. Síntesis de los derivados policatiónicos tetraalquilados de trehalosa **94**, **95**, **98** y **99**. Reactivos y condiciones: (a) 1-bromohexano ó 1-bromotetradecano, NaH, DMF, t.a.→ 60 ºC, 16 h, 57-73%; (b) $AlCl_3$, DCM, Et_2O, 40 ºC, 4 h, 41-82%; (c) I_2, TPP, imidazol, tolueno, 70 ºC, 5 h, 83-100%; (d) $HS(CH_2)_2NHBoc$, Cs_2CO_3, DMF, 60 ºC, 16 h, 100%; (e) 1:1 TFA-DCM, t.a., 15 min, 83-100%; (f) NaN_3, DMF, 70 ºC, Ar, 16 h, 86-88%; (g) TPP, THF, NH_4OH, 50 ºC, 16 h, 86-100%.

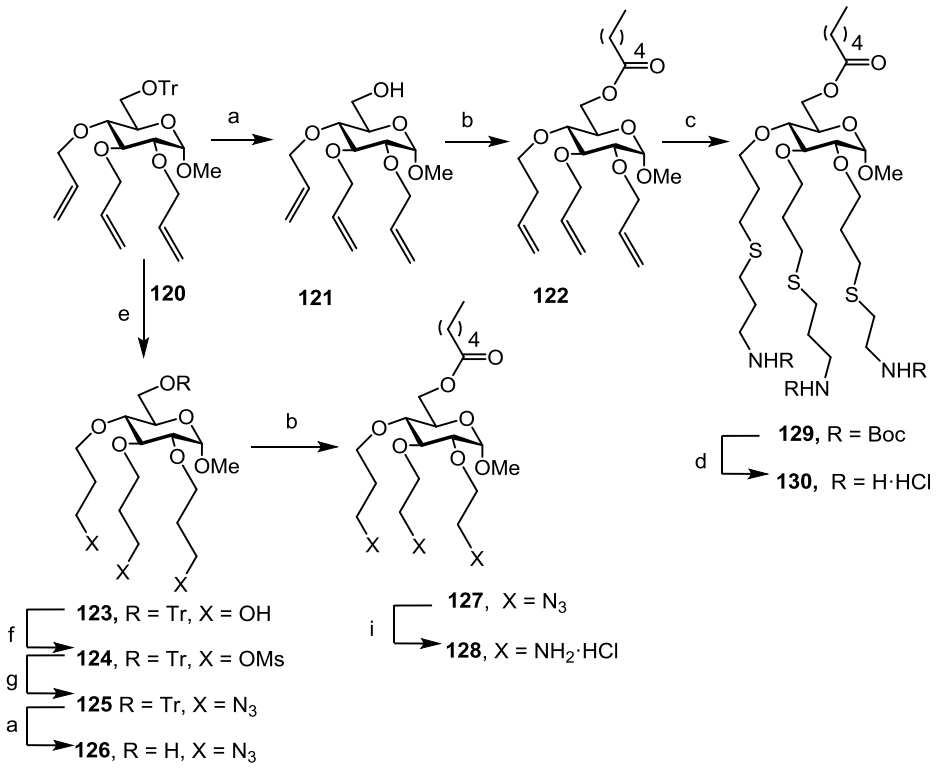
4.2.2. Sistemas tipo ''medusa''.

Resultados anteriores del grupo de investigación indicaron que los derivados catiónicos anfifílicos de β-ciclodextrina tipo ''medusa'' pueden adaptarse bien a la complejación y protección de DNA y comportarse como vectores de transfección con baja toxicidad modulando la anfiflia de la molécula.[23] Para obtener derivados con esta arquitectura sobre plataformas de glucosa y trehalosa hemos partido de los compouestos **120** y **133**, que se obtuvieron mediante alquilación exhaustiva con bromuro de alilo de los 6-*O*-tritil y 6,6'-di-O-tritil derivados correspondientes **171** y **173** (Esquema 4.12.)[i**Error! Marcador no definido.**]. La hidroboración-oxidación de los grupos alilo[24] (→**123**, **138**), seguida de mesilación (→**124**, **139**) y sustitución nucleofílica con azida de sodio condujo a las azidas tritiladas **125** y **140**. La hidrólisis posterior de los grupos tritilo con PTSA seguida de hexanoilación de las posiciones primarias permitió acceder a los derivados **127** y **136**. Finalmente, la reducción empleando las condiciones de Staudinger condujo a las aminas objetivo **128** y **137**, que fueron aisladas en forma de

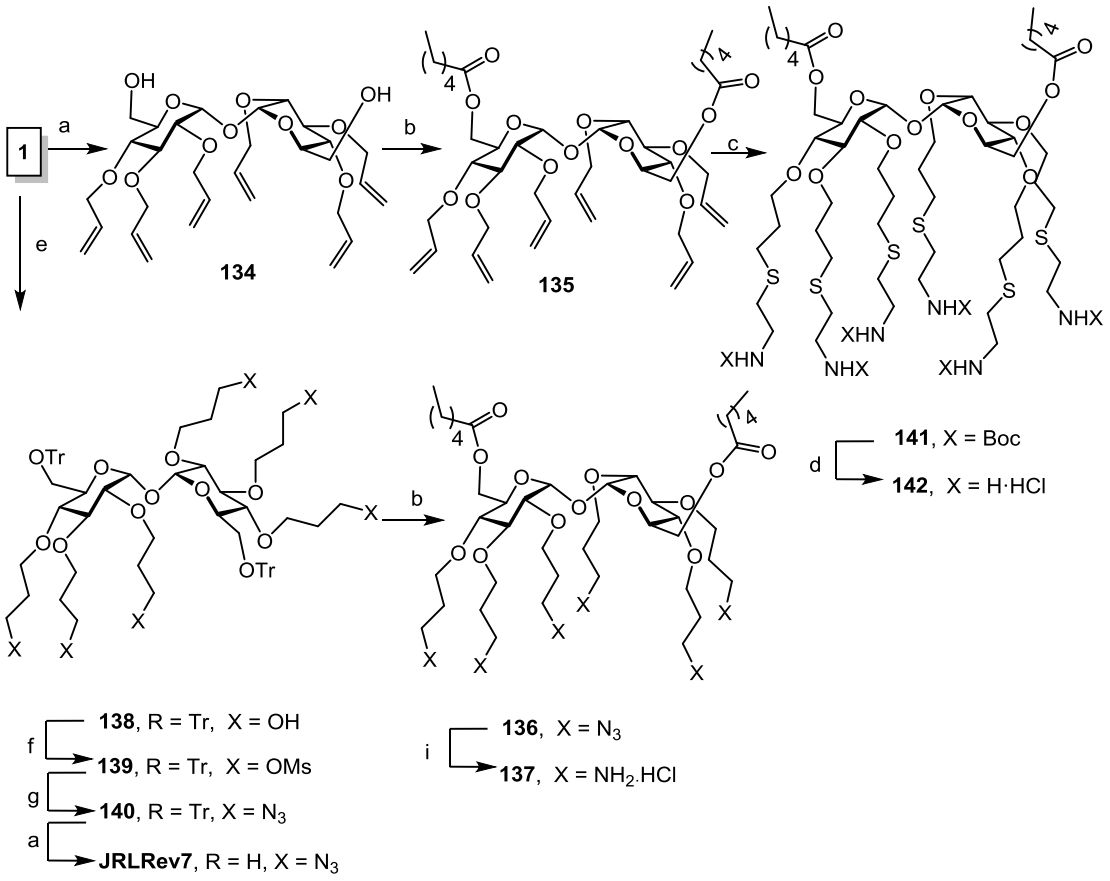
hidrocloruro tras liofilizar en presencia de HCl diluido, con buenos rendimientos. Por otra parte, a partir de los derivados alilados **122** y **135** y haciendo uso del acoplamiento fotoinducido entre tioles y alquenos (reacction "click" tiol-eno),[25] se han preparado los derivados de cisteamin-Boc **129** y **141**. La hidrólisis ácida posterior de los grupos carbamato (→**130**, **142**) seguida de liofilización permitió aislar los correspondientes hidrocloruros con excelentes rendimientos (Esquemas 4.13 y 4.14). Adicionelmante, se llevó a cabo la inserción de segmentos de tiourea en los sustituyentes de la cara secundaria (→ **132** y **144**) mediante acoplamiento nucleofílico con el isotiocianato protegido **168** y posterior hidrólisis ácida de los grupos carbamato (Esquema 4.15.).



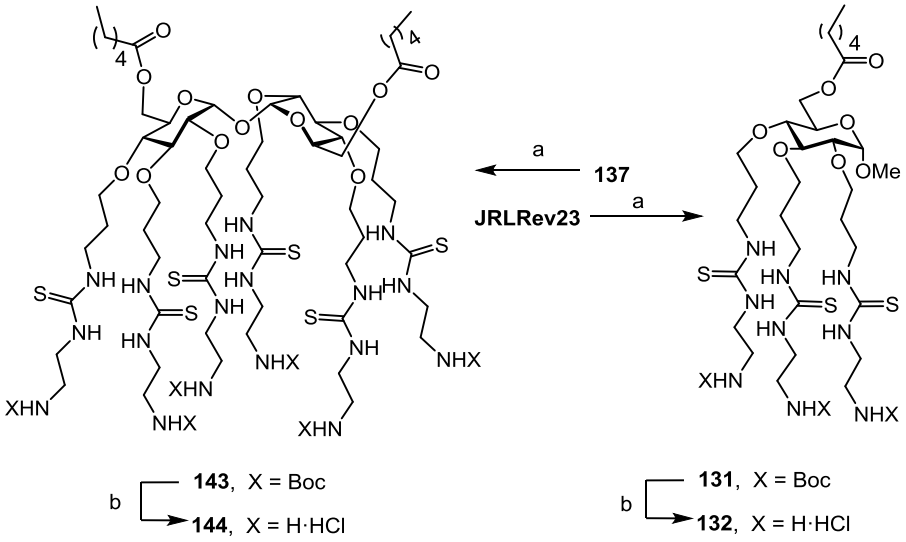
Esquema 4.12. Síntesis de los derivados de glucosa y trehalosa peralilados en la cara secundaria **120** y **133**. Reactivos y condiciones: (a) Bromuro de alilo, NaH, DMF, t.a., Ar, 16 h, 93%, 73%.



Esquema 4.13. Síntesis de los derivados anfifílicos catiónicos tipo "medusa" de glucosa **130 y 128**. Reactivos y condiciones: (a) PTSA, DCM-MeOH, t.a., 3 h, 89%; (b) anhidrido hexanoico, DMAP, DMF, t.a., 3 h, 74-93%; (c) $HS(CH_2)_2NHBoc$, t.a., hν (λ = 245 nm), 1 h, 95%; (d) 1:1 TFA-DCM, t.a., 15 min, 99%; (e) 9-BBN, THF, reflujo, 4 h, 73%; (f) MsCl, Et_3N, DCM, t.a., Ar, 30 min, 62%; (g) NaN_3, DMF, 70 ºC, Ar, 16 h, 86-88%; (g) TPP, THF, NH_4OH, 50 ºC, 16 h, 86-cuantitativo.



Esquema 4.14. Síntesis de los derivados anfifílicos catiónicos tipo "medusa de trehalosa **137** y **142**. Reactivos y condiciones: (a) PTSA, DCM-MeOH, t.a., 3 h, 85% (b) anhidrido hexanoico, DMAP, DMF, t.a., 3 h, 77-100% (c) $HS(CH_2)_2NHBoc$, t.a., hν (λ = 245 nm), 1 h, 93% (d) 1:1 TFA-DCM, t.a., 15 min, 99%. (e) 9-BBN, THF, reflujo, 4 h, 69%; (f) MsCl, Et_3N, DCM, t.a., Ar, 30 min, 98% (g) NaN_3, DMF, 70 ºC, Ar, 16 h, 98%; (i) TPP, THF, NH_4OH, 50 ºC, 16 h, 86-100%.



Esquema 4.15. Síntesis de los derivados catiónicos catiónicos tipo "medusa **132 y 144**. Reactivos y condiciones: (a) **168**, Et_3N, DCM, Ar, 16 h; (b) 1:1 TFA-DCM, t.a., 15 min, 99%.

Las estructuras de todos los derivados preparados se confirmó mediante microanálisis, espectrometría de masas (ESI-MS) y ^{1}H y ^{13}C RMN, confirmándose la presencia de un único sistema de spin consistente con simetría C_2 en el caso de los derivados de trehalosa (Figura 4.5).

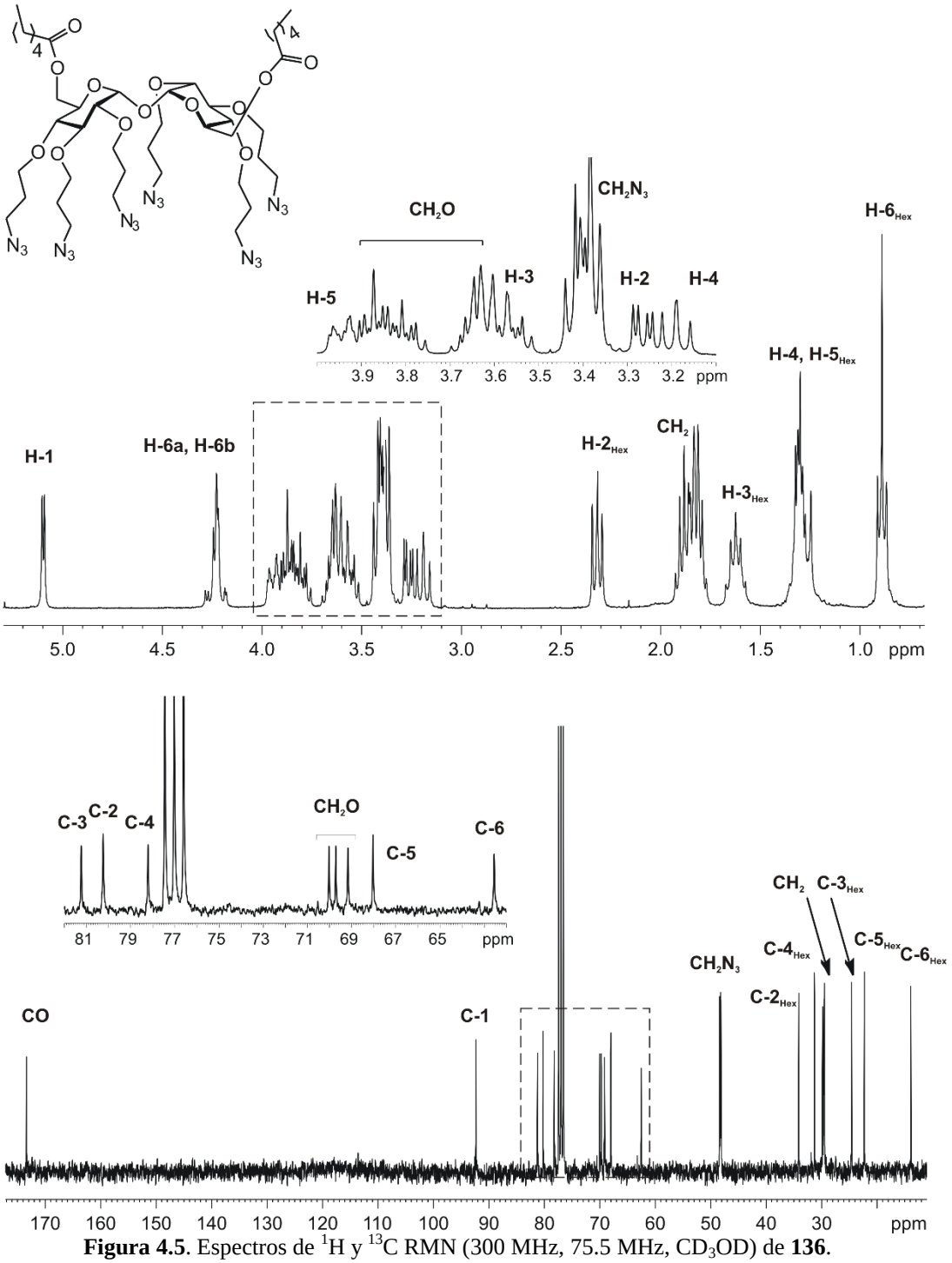


Figura 4.5. Espectros de ^{1}H y ^{13}C RMN (300 MHz, 75.5 MHz, CD_3OD) de **136**.

4.2.3. Estudio de las propiedades supramoleculares de agregación y autoensamblado.

Con objeto de analizar la influencia de las diferentes modificaciones estructurales en las propiedades de agregación en medio acuoso, se ha determinado concentración micelar crítica de todos los compuestos preparados, empleando un método basado en cambios de fluorescencia del pireno,[26] así como tamaño de los agregados resultantes y su potencial de carga superficial (potencial zeta – ζ) mediante DLS (Dynamic Light Scattering). Brevemente, en medio acuoso la molécula de pireno se incorpora al interior hidrófobo de los agregados, lo que induce cambios en su espectro de fluorescencia. Esto produce un desplazamiento del espectro de excitación (λ_{ex} 310-340 nm) al emplear una λ_{em} de 375 nm (Fig). La representación gráfica de la relación de intensidades de los espectros de fluorescencia I_{339}/I_{335} frente a la concentración empleada permite determinar la CMC de cada derivado (Fig 4.6.). Los valores de CMC, junto con los de tamaño hidrodinámico, potenciales zeta, índices de polidispersidad de los agregados se recogen en las Tablas 4.1 a 4.4, ordenados por familias.

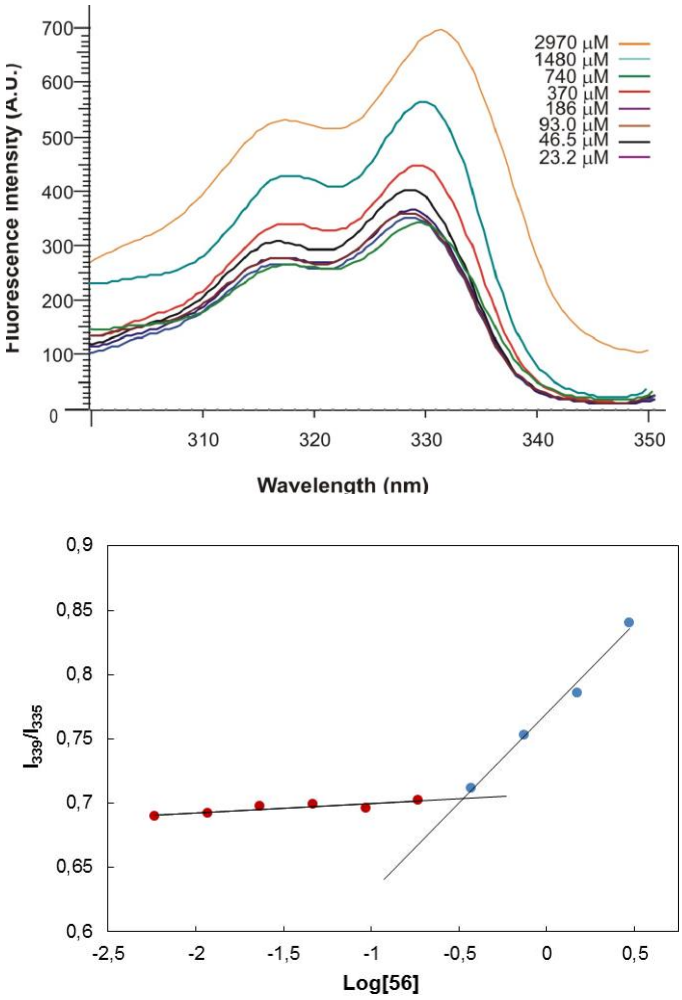


Figura 4.6. Determinación de la concentración micelar crítica (CMC) mediante fluorescencia.

Tabla 4.1 Concentraciones micelares críticas (μM), diámetros hidrodinámicos, desviaciones estándar, índices de polidispersidad y potencial ζ (mV) de los derivados catiónicos anfifílicos poliacilados tipo falda de glucosa.

Comp.	CMC (μM)	Tamaño (nm) ± SD	PI	Potencial ζ (mV) ±SD
11	458.5 ± 123.7	618.7 ± 282.9	0.513	33.8 ± 0.77
47	59.7 ± 10,5	111.7 ± 0.5196	0.26	52.4 ± 1.55
50	89.5 ± 20.8	105.2 ± 0.5774	0.223	46.3 ± 0.702
52	565 ± 34	247.8 ± 36.7	0.332	7.64 ± 0.665
54	134.1 ± 36.4	535.4 ± 83	0.468	19.1 ± 7.52
56	426,1 ± 159.6	176.6 ± 0,05	0.194	25.7 ± 7.17
58	129.7 ± 11.4	418.1 ± 44.65	0.371	38.7 ±5.61
60	33.8 ± 0.9	425 ± 47.11	0,425	22.3 ± 4.22
62	101.4 ± 0.49	847.2 ± 237.8	0.724	61.7 ± 2.33
64	112.1 ± 18.7	284 ± 32.4	0.362	41.4 ± 9.82
66	293.5 ± 13.0	205.4 ±130.9	0.645	6.91 ± 4.3

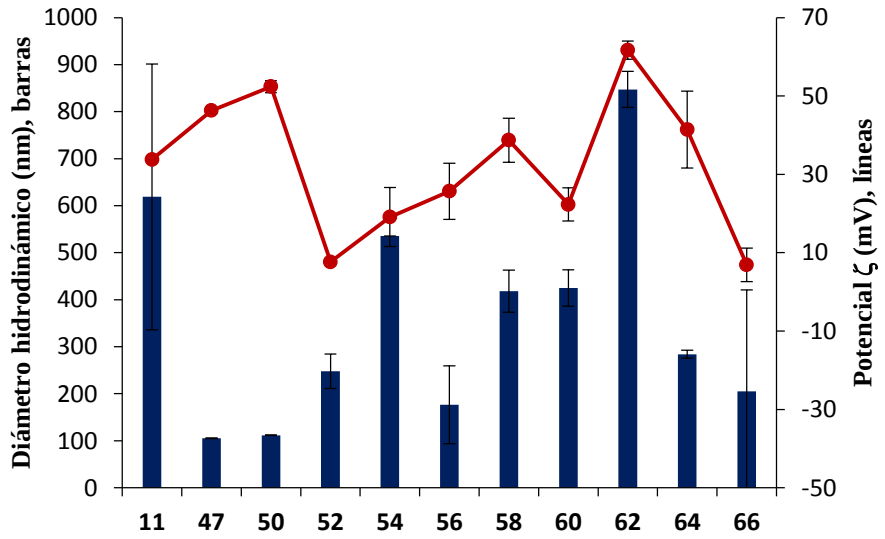


Tabla 4.2 Concentraciones micelares críticas (μM), diámetros hidrodinámicos, desviaciones estándar, índices de polidispersidad y potencial ζ (mV) de los derivados catiónicos anfifílicos poliacilados tipo falda de trehalosa.

Comp.	CMC (μM)	Tamaño (nm) ± SD	PI	Potencial ζ (mV) ±SD
70	97.7 ± 10	201.7 ± 38.41	0.343	45.4 ± 4.09
101	48.1 ± 16.50	183.4 ± 7.14	0.363	28.7 ± 4.10
103	57.4 ± 10.40	123.8 ± 8.32	0.246	44.7 ± 5.54
105	75.1 ± 30.0	688 ± 215.40	0.700	51.5 ± 6.34
107	350.5 ± 70.50	535.4 ± 83	0.468	19.1 ± 7.52
109	10.9 ± 2.10	202.9 ± 7.62	0.327	17.6 ± 2.00
111	360.5 ± 116.60	247.8 ± 36.7	0.332	44.8 ± 4.30
115	9.0 ± 1.27	505.7 ± 146	0.458	41.5 ± 6.51
117	3.35 ± 0,07	353.3 ± 24.24	0.473	70.7 ± 3.22
119	275 ± 9.2	469.5 ± 65.53	0.451	73.3 ± 14.80

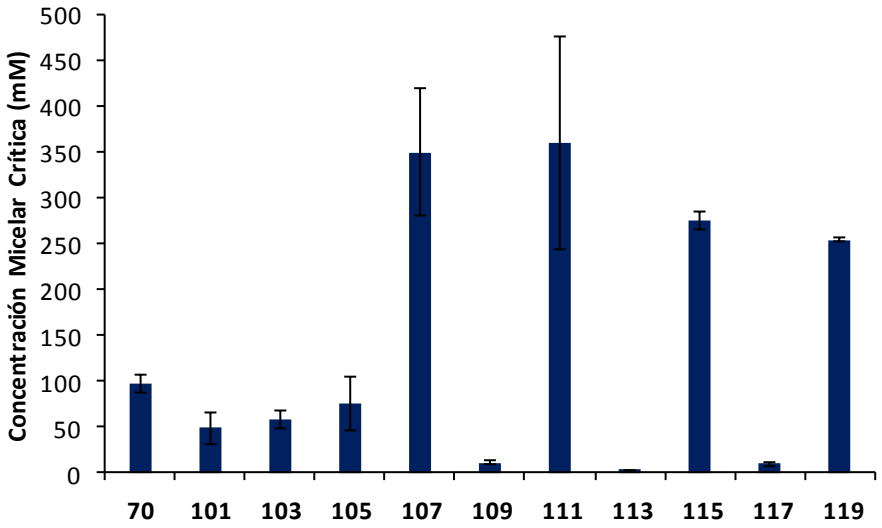


Tabla 4.3 Concentraciones micelares críticas (μM), diámetros hidrodinámicos, desviaciones estándar, índices de polidispersidad y potencial ζ (mV) de los derivados catiónicos polialquilados tipo falda de glucosa.

Comp.	CMC (μM)	Tamaño (nm) ± SD	PI	Potencial ζ (mV) ±SD
23	248 ± 57	169.6 ± 39.36	0.453	13.7 ± 2.,06
24	23.1 ± 2.3	137.6 ± 2.722	0.386	22.5 ± 0.351
27	195.8 ± 9.1	118.6 ± 2.603	0.428	18.3 ± 1.42

28	1.9 ± 0.2	168.5 ± 3.147	0.356	43 ± 2.72
37	1355 ±162	268.7 ± 5.37	0.151	2.4 ± 2.49
38	45.5 ±9.2	164.6 ± 0.643	0.287	23.6 ± 0.196
43	1475 ± 318	179.7 ± 2.138	0.166	12.2 ± 1.11
44	79.3 ± 14	69.44 ± 1.587	0.254	44.2 ± 1.5

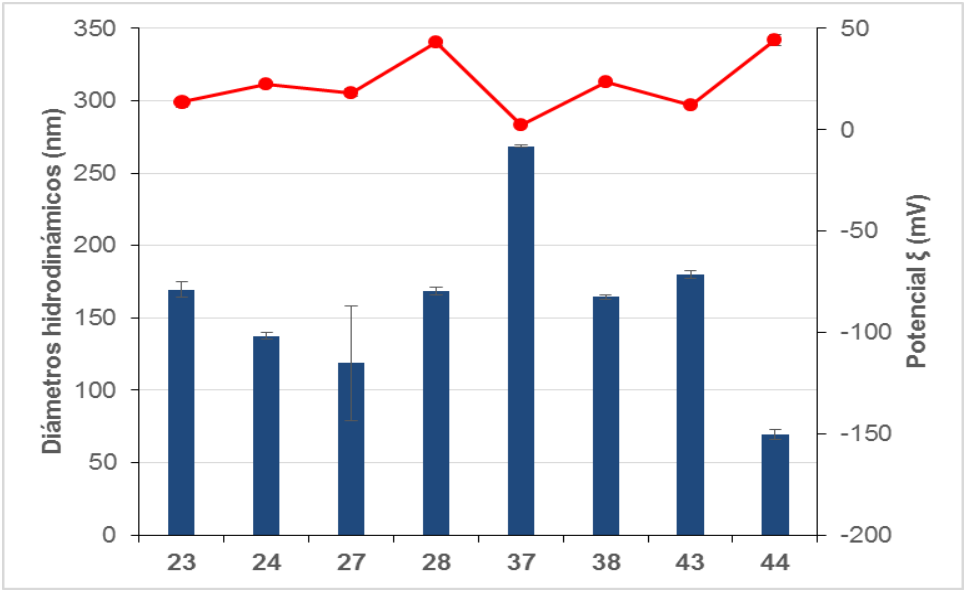


Tabla 4.4 Concentraciones micelares críticas (μM), diámetros hidrodinámicos, desviaciones estándar, índices de polidispersidad y potencial ζ (mV) de los derivados catiónicos polialquilados anfifílicos tipo falda de trehalosa.

Comp.	CMC (μM)	Tamaño (nm) ± SD	PI	Potencial ζ (mV) ±SD
80	8.3 ± 3.3	130.5 ± 2.02	0.31	46.1 ± 1.04
81	1,04 ± 0.2	123.2 ± 1.079	0.318	45 ± 3.95
84	2.24 ± 0.7	172.6 ± 13.96	0.218	5.65 ± 0.391
85	1.35 ± 0,24	179 ± 7.13	0.208	2.17 ± 0.32
94	80.6 ± 8.9	139.4 ± 9.99	0.4	3.77 ± 1.28
95	17 ± 10	142.5 ± 2.72	0.356	50.2 ± 3.85
98	285.5 ± 9.2	97.8 ± 1.318	0.223	22.3 ± 0.804
99	10 ± 0.9	164.1 ±6.786	0.505	10.2 ± 0.799

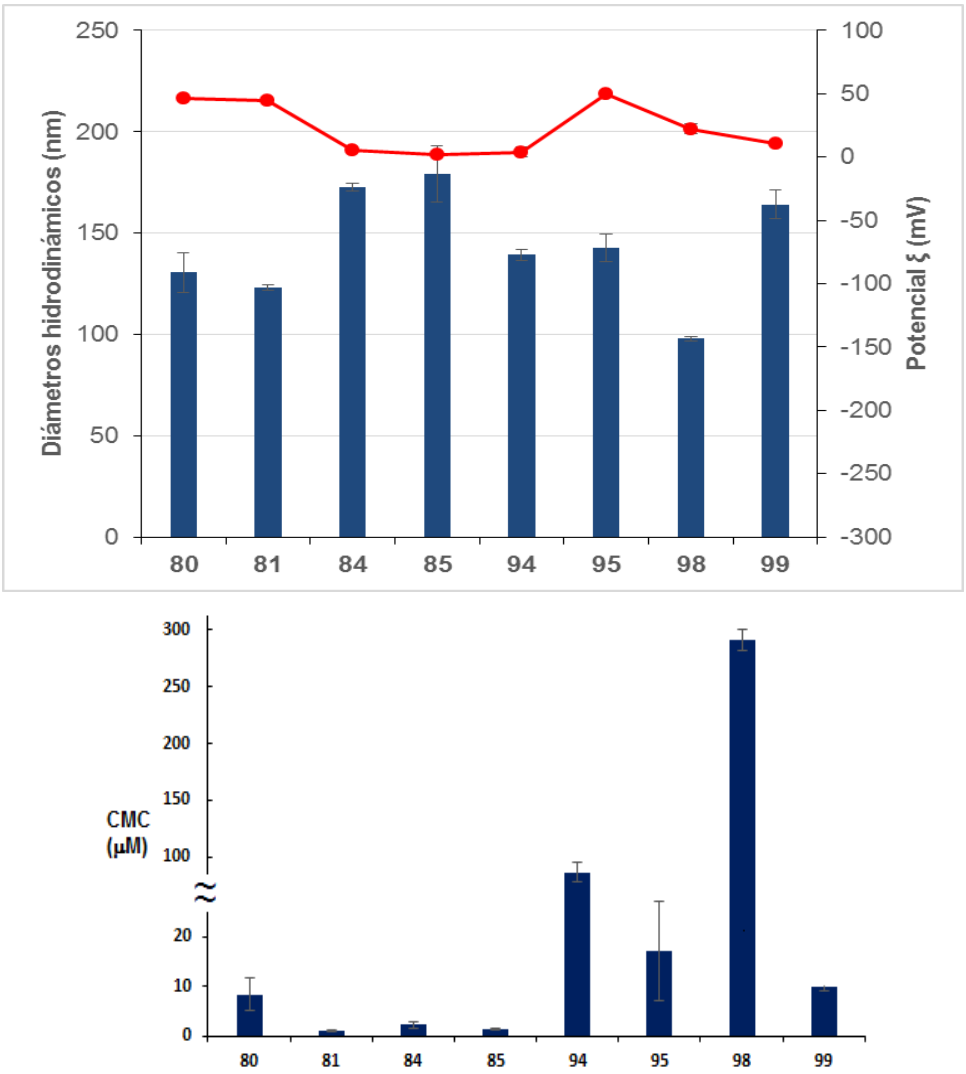


Figura 4.8. Concentraciones micelares críticas para los derivados policatiónicos alquilados.

En el caso de los derivados alquilados observamos una tendencia análoga. El aumento de la longitud de la cadena alquílica, de 6 a 14 carbonos produce en todos los casos una disminución significativa de la CMC, que alcanza hasta dos órdenes de magnitud (por ejemplo, 195.8 μM para **27** y 1.9 μM para **28**). Los derivados que conservan algún grupo hidroxilo sin alquilar muestran un aumento de la CMC respecto a sus homólogos peralquilados, tanto en los derivados de glucosa como en los de trehalosa.

Finalmente, en el caso los derivados tipo medusa, al presentar un balance hidrófilo-hidrófobo opuesto, con predominio de grupos cargados, se observan concentraciones micelares críticas muy elevadas (Figura 4.9), en el rango milimolar, que indican una baja tendencia a la autoasociación.

Por su parte, los derivados en fase reversa (tipo medusa), al presentar un balance hidrófilo-hidrófobo diferente, con predominio de grupos cargados y un déficit en cadenas acílicas, presentan concentraciones micelares críticas muy elevadas (Figura 4.9), cercanas o superando el

orden milimolar, lo que no les otorga propiedades óptimas de autoensamblaje y, en efecto, no se observa formación de nanopartículas en ninguna de ellas y los bajos potenciales ζ son indicativos de inestabilidad y alto grado de floculación.

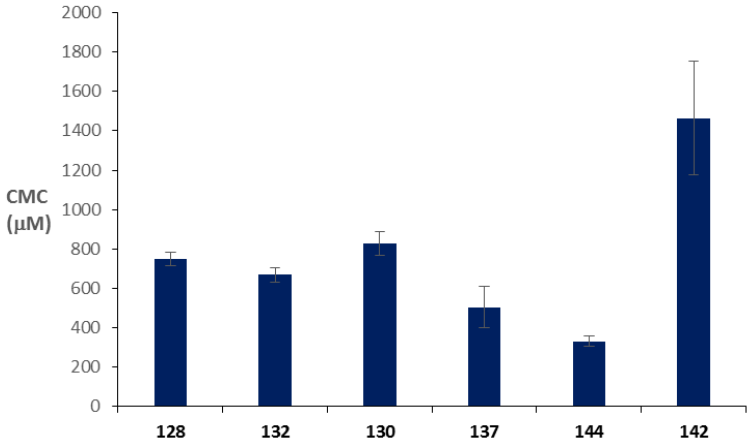


Figura 4.9. Concentraciones micelares críticas para los derivados policatiónicos tipo medusa.

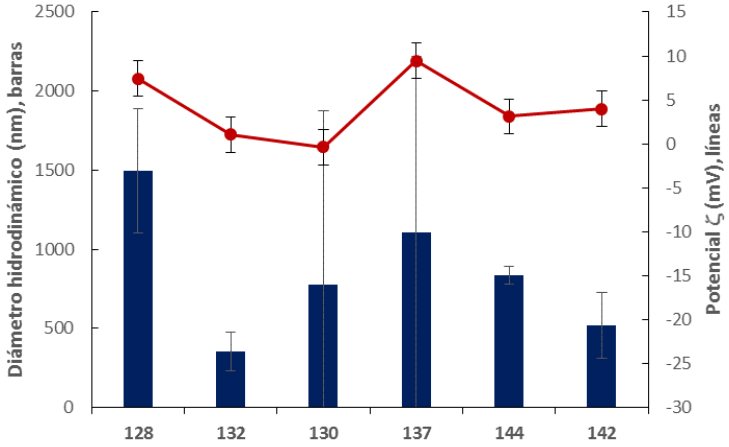


Figura 4.10. Concentraciones micelares críticas para los derivados policatiónicos tipo medusa.

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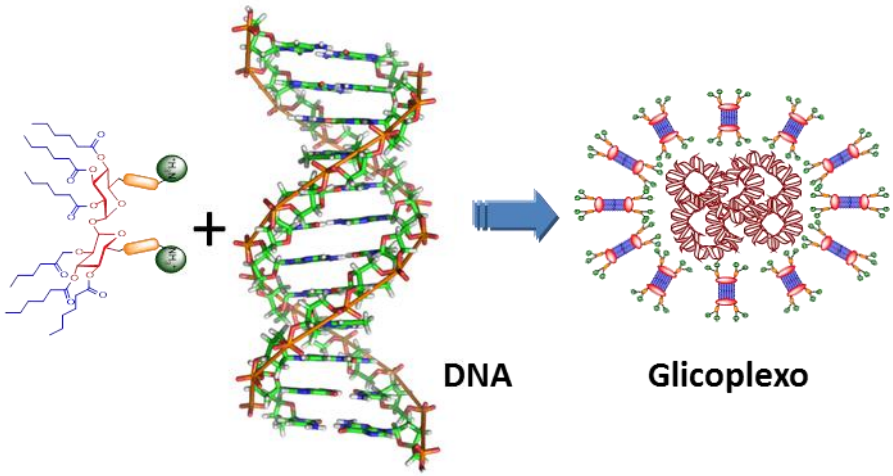
Capítulo 5

Evaluación de derivados catiónicos de glucosa y trehalosa como vectores de genes en comparación con estructuras macrocíclicas.

Abstract: All ‘’skirt-like’’ and ‘’medusa-like’’ glucose and trehalose policathionic amphiphilic derivatives were evaluated as gene vectors for gene therapy. Initially, the ability of all glycoderivatives to interact with DNA to form stable ‘glycoplexes’ was tested. Subsequently, transfection efficiency and cell viability of each vector was evaluated. In order to determine if ‘’facial amphiphilicity’’ is a prerrequisite for optimized gene delivery capabilities, a comparative study with macrocyclic polycationic amphiphilic cyclotrehalans (paCTs) was carried out.



5. Evaluación de derivados catiónicos anfifílicos de glucosa y trehalosa como vectores de genes en comparación con estructuras macrocíclicas (ciclotrehalanas).

5.1. Introducción

La funcionalización de la molécula de trehalosa en las posiciones primarias y secundarias de manera diferenciada permite acceder a compuestos que, en principio, están dotados de anfifilicidad facial. Sin embargo, es posible que, a pesar de la rigidez del núcleo disacarídico, la estructura no proporcione una orientación espacial suficientemente definida de los dominios catiónico e hidrófobo, o que la relación entre posiciones primarias y secundarias en la molécula no permita alcanzar un balance hidrófilo-hidrófobo adecuado para esta aplicación. En el marco de un proyecto de investigación más amplio, se planteó realizar un estudio de estructura-capacidad de transfección incluyendo, además de los derivados policatiónicos anfifílicos de metil α-D-glucopiranósido y de α,α'-trehalosa preparados en esta Tesis, una nueva serie de análogos ciclooligosacarídicos resultantes de la macrociclización de dos subunidades del disacárido a través de sus posiciones primarias. Este tipo de compuestos, denominados ciclotrehalanas (CTs)[1,2,3,4,5,6] pueden obtenerse con buenos rendimientos a partir de precursores funcionalizados con grupos isotiocianato y amina. La eficacia del método se debe, posiblemente, a las características geométricas favorables de la molécula de α,α'-trehalosa que orienta adecuadamente las posiciones primarias con los grupos reactivos isotiocianato y amino hacia la misma región del espacio (Figura 5.1.). Tras la formación del primer puente de tiourea, la reacción intramolecular está fuertemente favorecida frente al acoplamiento intermolecular para dar oligómeros lineales.[1,3]

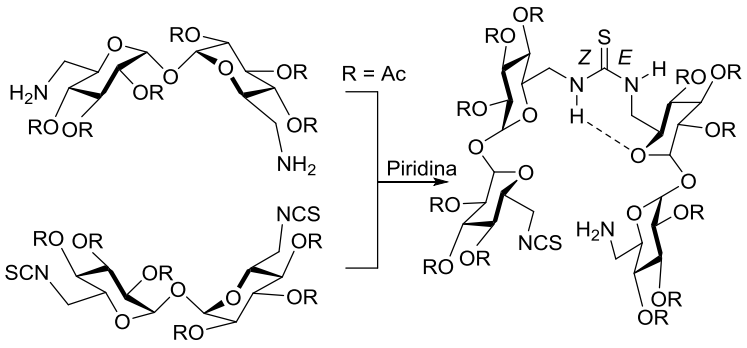


Figura 5.1. Disposición espacial de los dímeros de trehalosa tras la formación del primer puente de tiourea.

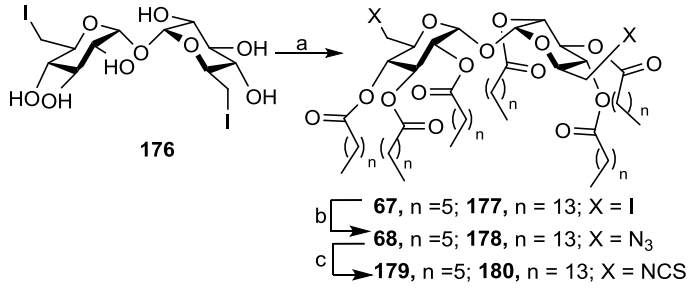
Como sucede con las ciclodextrinas (CDs), las CTs presentan los grupos hidroxilo expuestos en regiones opuestas. Sin embargo, mientras que en las CDs es necesario implementar estrategias de funcionalización facial regioselectiva para diferenciar ambas caras, en el caso de las CTs es posible instalar los grupos funcionales sobre las unidades de trehalosa con anterioridad a la generación del macrociclo, lo que ofrece una gran flexibilidad. Los compuestos finales tendrán por tanto dos caras diferenciadas cada una de las cuales incorpora seis antenas hidrofóbicas y seis cabezas catiónicas ancladas a hidroxilos secundarios. De esta manera, en paralelo a esta Tesis, se ha preparado una serie de CTs diméricas (ciclotetrasacarídicas) que difieren en factores como la densidad de carga y la flexibilidad del clúster catiónico, y que están estructuralmente relacionadas con los derivados disacarídicos de α,α',-trehalosa sintetizados en esta Tesis. Sobre los nuevos derivados preparados se ha llevado a cabo un estudio de sus propiedades de agregación (CMCs, tamaños hidrodinámicos y carga superficial). Además, se han evaluado las propiedades de complejación, protección de ADN y transfección empleando las líneas celulares COS7 y HepG2 en colaboración con la profesora Concepción Tros de Ilarduya, de la Universidad de Navarra.

5.2. Resultados y Discusión

5.2.1. Preparación de ciclotrehalanas anfifílicas policatiónicas (paCTs).

La síntesis de las CTs policatiónicas anfifílicas descritas en este apartado ha sido completada dentro del grupo de investigación por la Lcda. Eva Mª Aguilar Moncayo y el Dr. José Luis Jiménez Blanco. Se ha seguido una estrategia convergente con una etapa clave de macrociclación basada en el acoplamiento intermolecular entre un derivado de tipo 6,6'-diisotiocianato y otro de tipo 6,6'-diamina de la trehalosa, con los grupos hidroxilos secundarios convenientemente funcionalizados. Como precursor de la cara lipofílica se ha utilizado el correspondiente diisotiocianato hexaacilado con cadenas de hexanoilo o tetradecanoilo **179** o **180**, y como precursor del dominio policatiónico el derivado de 6,6'-diamino-6,6'-didesoxitrehalosa con cadenas de 2-*N*-*tert*-butoxicarbonilaminoetiltiopropilo en las posiciones secundarias **181**.

La preparación de los diisotiocianatos **179** y **180** se llevó a cabo mediante un proceso en tres etapas que parte de la 6,6'-dideoxi-6,6'-diiodo-α,α'-trehalosa.[7] La esterificación con el anhídrido del ácido correspondiente en presencia de *N*,*N*-dimetilaminopiridina (DMAP; →**67**, **177**) seguida de sustitución nucleofílica de los grupos yodo por anión azida[7] (→**68**, **178**) e isotiocianación usando el sistema TPP-disulfuro de carbono,[8] condujo a los derivados objetivo con buenos rendimientos (Esquema 5.1).



Esquema 5.1. Síntesis de los diisotiocianatos hexanoilado y miristoilado derivados de trehalosa **179** y **180**. Reactivos y condiciones: (a) anhídrido hexanoico (mirístico), DMAP, DMF,

8 h, 76% (41%). (b) NaN_3, DMF, 40-55 ºC, 12 h, 72% (cuantitativo). (c) TPP/CS_2, DCC, DCM, t.a., 24 h, 96% (82%).

En la Figura 5.2 se muestran los espectros de ^{1}H y ^{13}C RMN (300, 75.5 MHz) del diisotiocianato **179**, donde se observa la señal a 136.3 ppm, característica del grupo funcional isotiocianato.

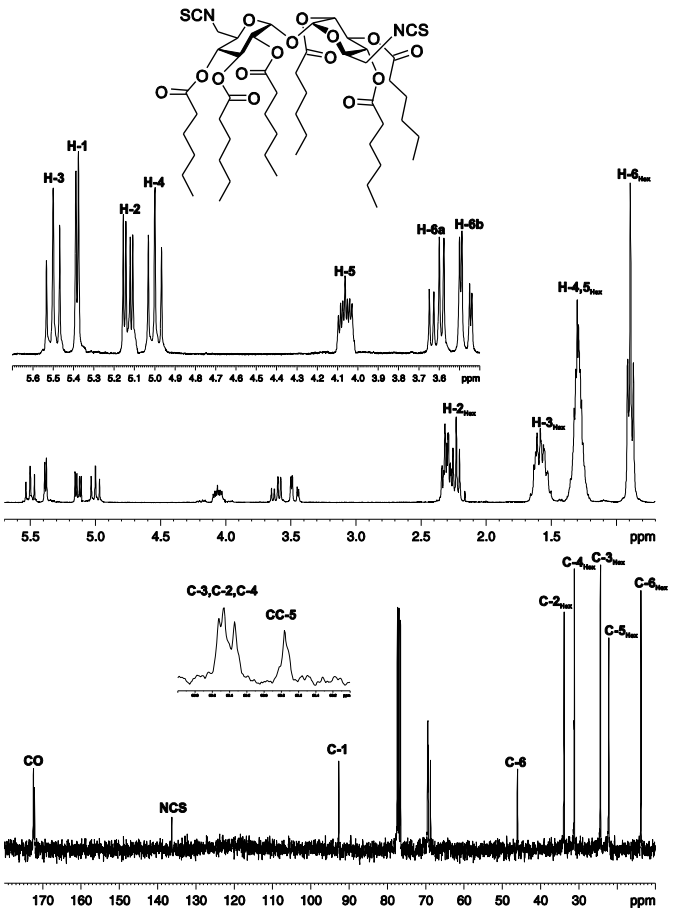
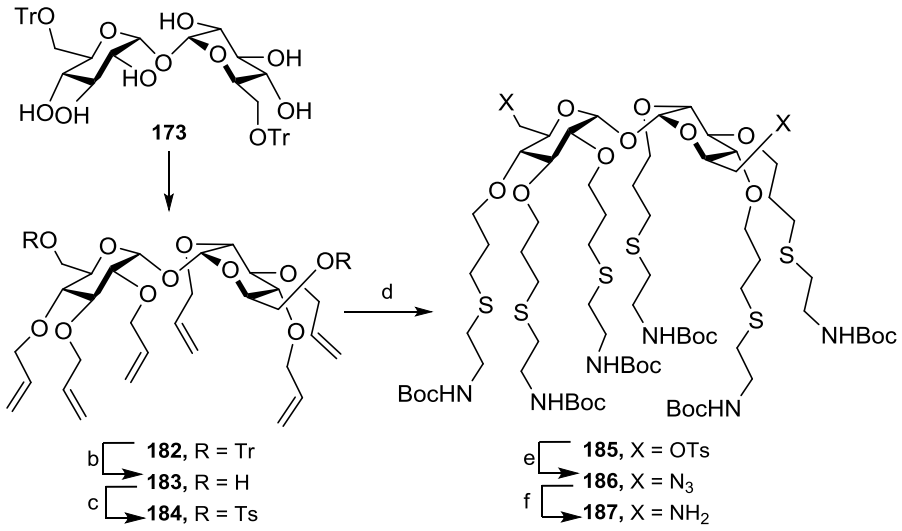


Figura 5.2. Espectros de ^{1}H y ^{13}C RMN (300 y 75.5 MHz, $CDCl_3$) de **179.**

La diamina **181** se obtuvo a partir de la 6,6'-di-*O*-tritil-α,α'-trehalosa[9] siguiendo la secuencia de reacciones que se muestra en el Esquema 5.2. En primer lugar, se llevó a

cabo la alilación de los hidroxilos situados sobre esta cara de la trehalosa ditritilada (→**182**),[10] seguida de la hidrólisis ácida de los grupos tritilo con ácido *p*-toluensulfónico (PTSA) (→**183**)[11] y la tosilación de las posiciones primarias (→**184**).[12] La posterior adición radicalaria la N-Boc-cisteamina a los dobles enlaces irradiando con luz UV (254 nm) en MeOH desgasificado, bajo Argón, condujo al hexacarbamato **185**.[13] La incorporación de los grupos amino a las posiciones primarias de la trehalosa se llevó a cabo mediante desplazamiento nucleofílico de los grupos tosilo por azida sódica (→**186**) y reducción de Staüdinger de la diazida resultante con TPP[7] (→**187**).



Esquema 5.2. Síntesis de la 6,6'-diamina policatiónica derivada de trehalosa. Reactivos y condiciones:(a) bromuro de alilo, NaH, DMF, 12 h, 76%. (b) PTSA, DCM-MeOH 1:1, t.a., 3 h, 83%. (c) TsCl, DMAP, DCM, t.a., 24 h, 70%. (d) $HS(CH_2)_2NHBoc$, MeOH, hν, 89%. (e) NaN_3, DMF, 56 ºC, Ar, 80%. (f) TPP, THF, t.a., 30 min, NH_4OH, 12 h, 98%.

Las estructuras de los compuestos **185-187** se confirmaron mediante espectroscopia de IR, RMN, espectrometría de masas y análisis elemental. Los espectros de ^1H y ^{13}C RMN demostraron la homogeneidad de los derivados. En la Figura 5.3 se muestran los espectros de **185** con las señales características de los anillos aromáticos de los grupos

tosilo (7.77 y 7.34 ppm) y del grupo cisteaminilo protegido como Boc a δ 5.08, 3.28 y 2.61 ppm.

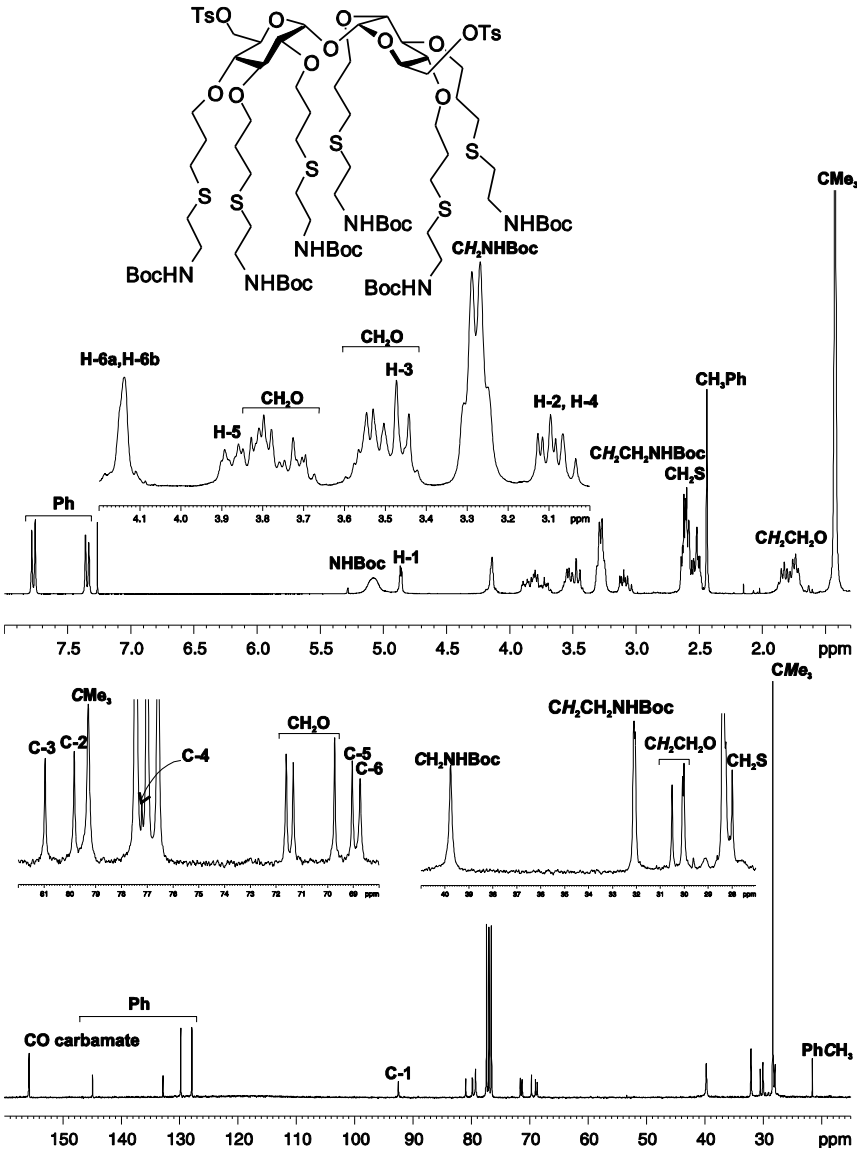
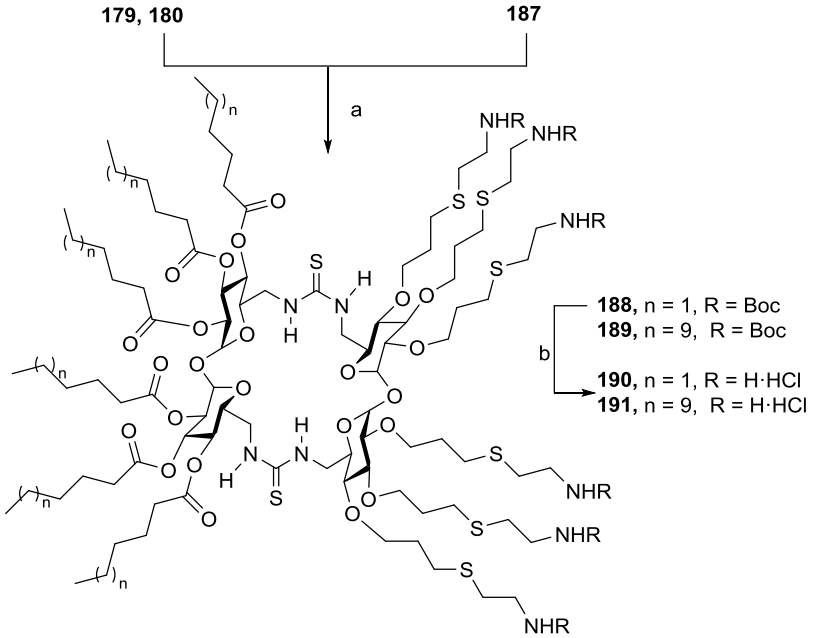


Figura 5.3. Espectros ^{1}H y ^{13}C RMN (300 y 75.5 MHz, $CDCl_3$) de **185.**

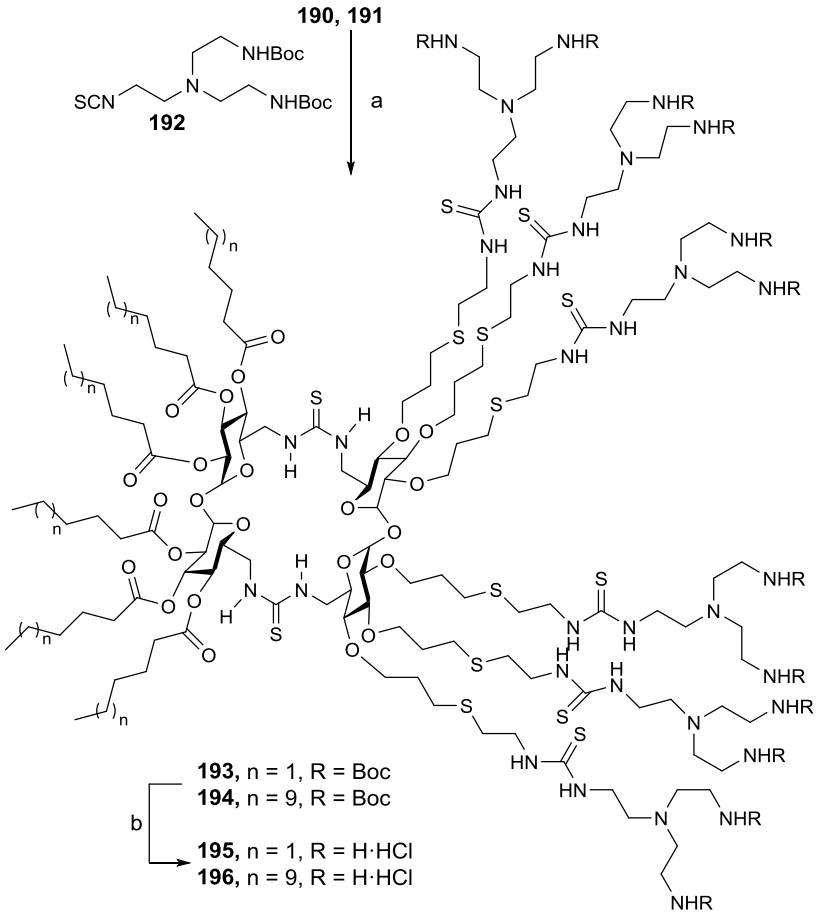
La síntesis de las ciclotrehalanas anfifílicas policatiónicas **190** y **191** se llevó a cabo siguiendo una estrategia convergente basada en el acoplamiento de los diisotiocianatos **179** y **180** con la diamina **187** en piridina a 40 ºC (→**188, 189**), seguido de desprotección de los grupos carbamato con TFA en diclorometano (Esquema 5.3). Los rendimientos obtenidos en la primera etapa son excelentes (81 y 76%) para una ciclación bimolecular.



Esquema 5.3. Síntesis de las ciclotrehalanas policatiónicas anfifílicas **190** y **191**. Reactivos y condiciones: (a) Piridina, 40 ºC, 8 h, 81% (76%). (b) 1:1 TFA-DCM, t.a., 30 min, cuantitativo.

Con objeto de acceder a derivados con una mayor densidad de grupos protonables, las ciclotrehalanas anfifílicas hexacatiónicas **190** y **191** se hicieron reaccionar con el isotiocianato dendrítico (**192**)[14,15] en piridina a 40 ºC y en presencia de DMAP (→**193, 194**)[16]. Tras purificación por cromatografía en columna de los correspondientes aductos de tioureas, la hidrólisis ácida de los grupos carbamatos con TFA en diclorometano condujo a las dodecaaminas **195** y **196** con excelentes rendimientos. Los compuestos

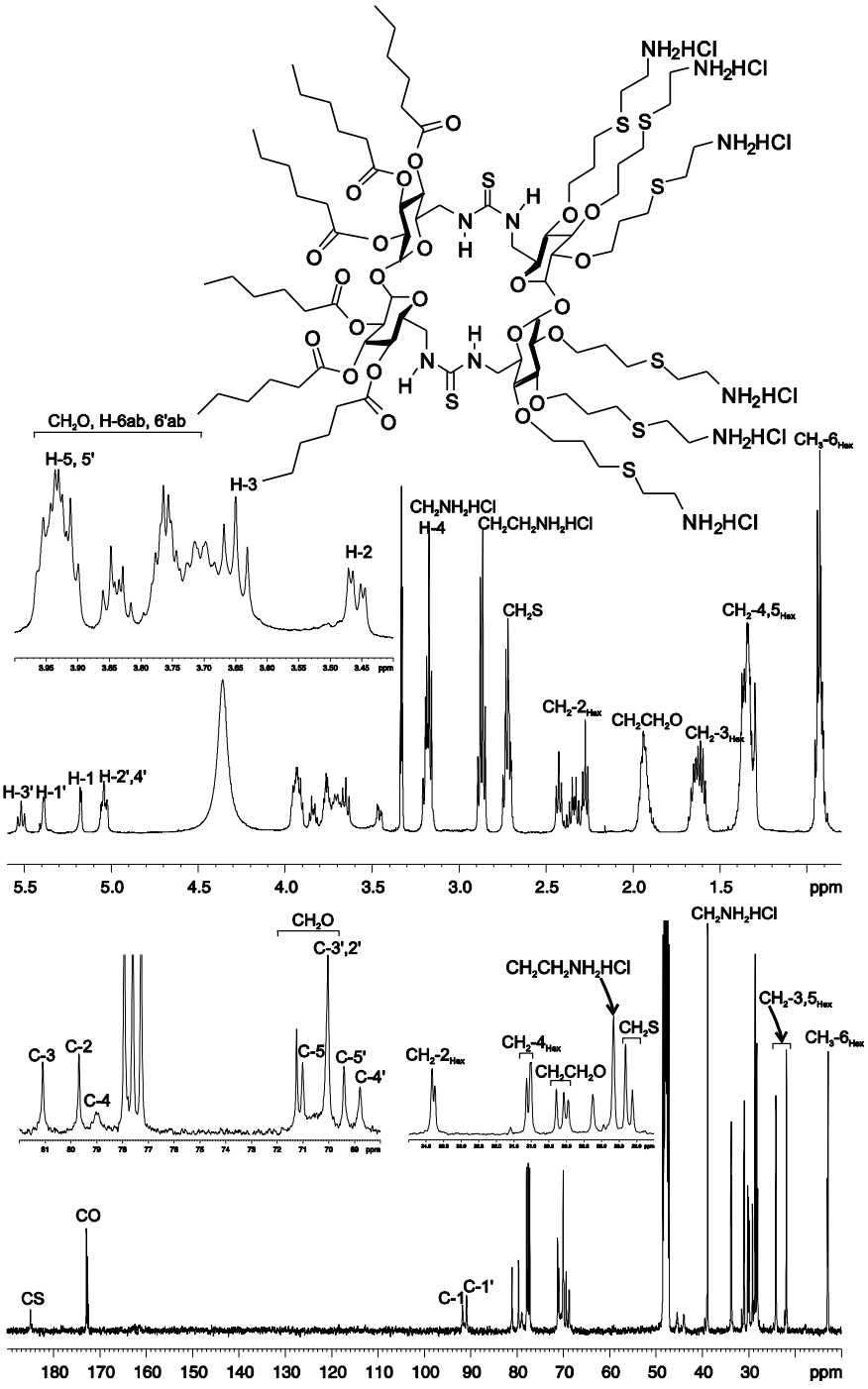
finales fueron aislados en forma de los correspondientes hidrocloruros tras liofilización con HCl diluido (Esquema 5.4).



Esquema 5.4. Síntesis de las ciclotrehalanas policatiónicas anfifílicas **195** y **196**. Reactivos y condiciones: (a) Piridina, DMAP, 40 ºC, 12 h, 75%(/99). (b) 1:1 TFA-DCM, t.a., 30 min, cuantitativo.

Los espectros de RMN y ESI-MS (Figura 5.4) permiten confirmar la estructura y homogeneidad de las hexatioureas dendríticas. La presencia de dos eje de simetría binarios perpendiculares determina que sólo se observen dos juegos de señales, que en el

caso de los protones de azúcar se encuentran entre 5.6-3-1 ppm y en el caso de los carbonos correspondientes a 95-65 ppm. Los espectros de RMN se llevaron a cabo a alta temperatura para evitar el ensanchamiento de señal debido al equilibrio conformacional lento de los enlaces HN─(C=S) de los grupos tiourea.[1,3,4]


NH2HCl
CH2O, H-6ab, 6'ab
H-5, 5'
H-3
H-2
CH2NH2HCl
H-4
CH2CH2NH2HCl
CH2S
CH2-4,5Hex
CH3-6Hex
CH2-2Hex
CH2CH2O
CH2-3Hex
H-3'
H-1'
H-1
H-2',4'
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
ppm
CH2O
C-3',2'
C-3
C-2
C-4
C-5
C-5'
C-4'
CH2-3,5Hex
CH2-4Hex
CO
CS
C-1
C-1'
180
170
160
150
140
130
120
110
100
90
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70
60
50
40
30

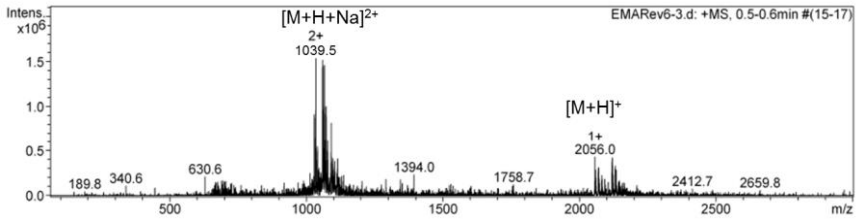
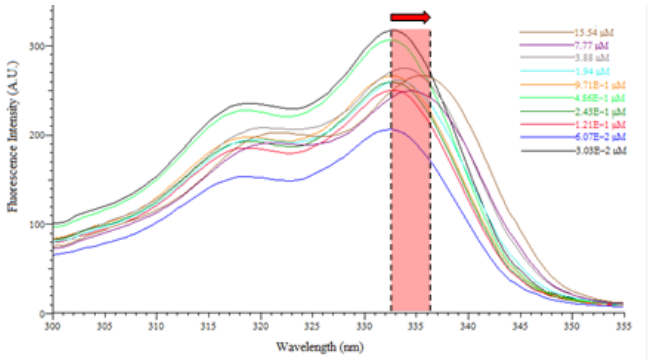


Figura 5.4. Espectros de ^{1}H y ^{13}C RMN (500 y 100.6 MHz, 6:1CD_3OD-$CDCl_3$, 333 K) y de EM-ESI de **190.**

5.2.2. Evaluación de las propiedades de autoorganización de las paCTs.

Para la evaluar la anfifilicidad y la capacidad de autoorganizarse en medio acuoso de las CTs se han realizado valoraciones fluorimétricas utilizando pireno como sonda fluorescente[17] con objeto de determinar la concentración micelar crítica (CMC). Las cuatro ciclotrehalanas anfifílicas policatiónicas se autoorganizan alrededor de las moléculas de pireno, obteniéndose valores de CMCs entre 1.2-3.3 μM para los compuestos **190**, **191** y **196** (Tabla 5.1), mientras que el valor obtenido para **195** es un orden de magnitud mayor (21 μM). Esta diferencia es previsible y puede atribuirse al mayor peso del dominio hidrofílico frente al hidrofóbico en este último compuesto.



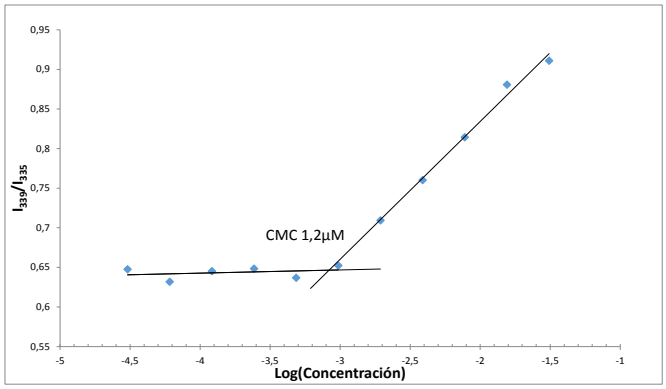


Figura 5.5. Determinación de la concentración micelar crítica del compuesto **191**. a) Espectros de excitación de la fluorescencia de pireno (λem 375 nm) en agua en presencia de **191**. (b) Determinación de CMC de **191**.

Tabla 5.1. Concentración micelar crítica (µM), tamaño hidrodinámico (nm), potencial ζ (mV) e índices de polidispersidad (PI) de las CTs anfifílicas **190**, **191**, **195** y **196.**

Comp.	CMC (µM)	[Conc.] (µM)	Tamaño (nm)	Potencial ζ (mV)	PI
190	3.3	5	194.4 ± 8.39	39.9 ± 3.35	0.466
		50	262.0 ± 12.33	41.9 ± 3.70	0.512
195	21.0	50	437.9 ±32.81	51.1 ± 1.76	0.688
		500	148.4 ± 0.78	65.8 ± 2.68	0.507
191	1.2	5	185.6 ± 22.62	55.1 ± 1.03	0.395
		50	481.6 ± 25.04	35.9 ± 0.10	0.371
196	1.3	5	220.6 ± 70.85	41.9 ± 5.55	0.447
		50	285.9 ± 62.05	48.5 ± 2.50	0.575

Para el estudio de las propiedades de autoorganización de estos compuestos se han preparado nanopartículas utilizando el método de hidratación de película fina descrito en los métodos generales. Las medidas del diámetro hidrodinámico, polidispersidad y potencial ξ de las nanopartículas obtenidas se llevaron a cabo utilizando la técnica de dispersión dinámica de la luz (DLS). Las concentraciones utilizadas para estas medidas estaban en el mismo orden y en un orden de magnitud superior al de las concentraciones

micelares críticas. Los resultados obtenidos, recogidos en la tabla 5.1, indican la formación de poblaciones de agregados de carga superficial positiva y de tamaño submicrométrico (Figura 5.6). Sin embargo los índices relativamente altos de polidispersidad indican que los compuestos no tienen tendencia a organizarse de manera ordenada en ausencia de ADN. Este tipo de comportamiento es análogo al observado con anterioridad para los derivados policatiónicos anfifílicos de ciclodextrinas.

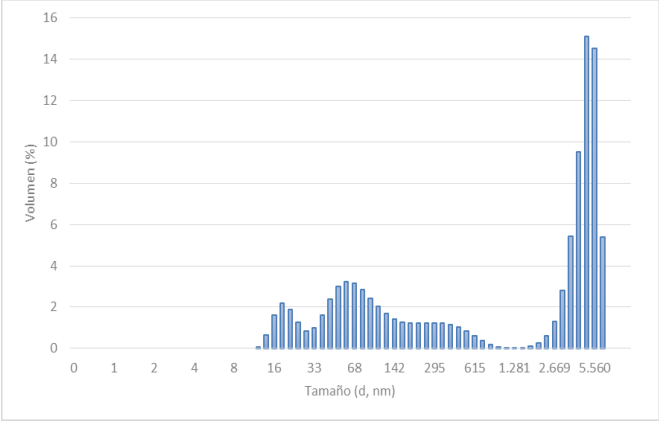


Figura 5.6. Distribución de tamaños de partícula en %volumen determinado por DLS para **190** formulado a 5 μM en H_2O.

5.2.3. Estudio comparativo de las interacciones con ADN de los derivados anfifílicos policatiónicos de glucosa, trehalosa y ciclotrehalana.

Electroforesis en gel de agarosa

La capacidad de los derivados catiónicos anfifílicos de metil α-D-glucopiranósido (Figura 5.7a) y de α,α'-trehalosa (Figura 5.7b), así como de las ciclotrehalanas **190**, **191**, **195, 196**, para complejar y proteger el ADN se ha estudiado en colaboración con la profesora Concepción Tros de Ilarduya, de la Universidad de Navarra. Para ello se prepararon los correspondientes complejos de los derivados anfífilicos con el plásmido que codifica la luciferasa eFLuc (pCpG-hCMV-SPEC-eFLuc, 4640 pares de bases)[18] y se

llevaron a cabo experimentos de electroforesis en gel de agarosa empleando valores de relación entre los nitrógenos protonables en el derivado poliamínico y los grupos fosfatos en el plásmido (N/P) de 5 y 10 (60 μM de fosfato), y GelRed^{TM} como agente intercalante fluorescente. Las formulaciones se prepararon por adición, sobre una disolución del pDNA en tampón HEPES (20 mM, pH 7.4), de la cantidad correspondiente de derivado anfifílico para cada valor de N/P. Se utilizó ADN plamídico libre como control negativo.

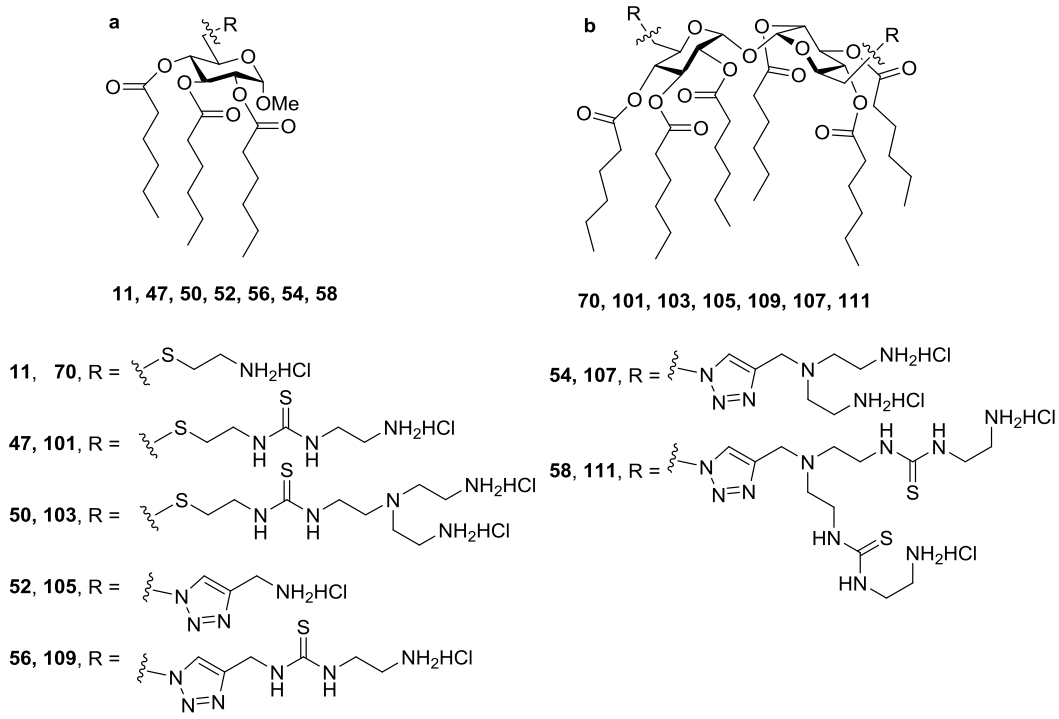


Figura 5.7. (a) Estructuras de los metil glucósidos anfifílicos catiónicos **11**, **47**, **50**, **52**, **56**, **54** y **58.** (b) Estructuras de trehalosas anfifílicas catiónicas **70**, **101**, **103**, **105**, **109**, **107** y **111**.

En la Figura 5.8 se muestran los resultados de los experimentos de migración electroforesis en gel de agarosa de los complejos de ADN con los derivados anfifílicos de glucosa y trehalosa. Todos los derivados protegen eficazmente el material génico a

valores de N/P 5 salvo **11** y **70**, en los que los grupos protonables se localizan en el segmento de cisteamina. Posiblemente, la proximidad de los grupos amonio a la plataforma sacarídica y a las cadenas lipófilas, junto con la ausencia de grupos tiourea que pudieran contribuir a la complejación de los aniones fosfatos mediante enlaces de hidrógeno, impiden una interacción eficaz con el ADN.[19] Los complejos de los derivados **54**, **58**, **107** y **111** muestran una nula accesibilidad del agente intercalante al material génico, lo que indica que en estos casos el ADN se encuentra perfectamente protegido en los correspondientes complejos. A valores de N/P 10 todos los compuestos complejan y protegen totalmente el ADN.

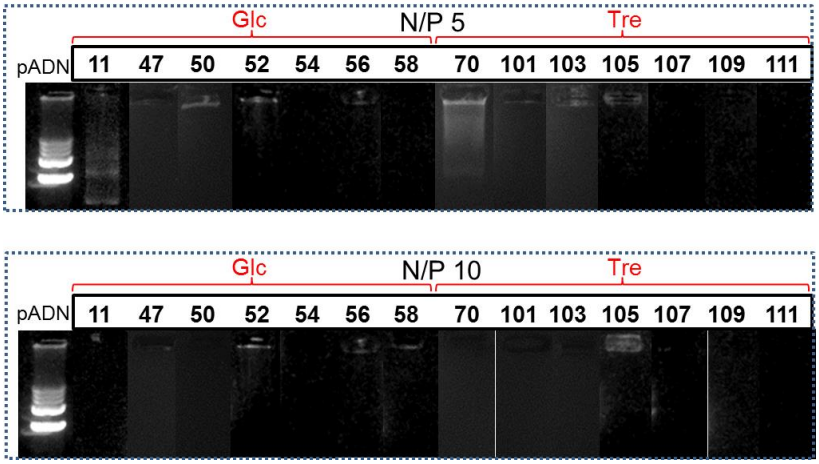


Figura 5.8. Electroforesis en gel de agarosa de las formulaciones de pADN y los derivados anfifílicos policatiónicos de glucosa y trehalosa a valores de N/P 5 y 10.

Los resultados de electroforesis en gel de agarosa de los complejos de pADN con las ciclotrehalanas anfifílicas policatiónicas **190**, **191**, **195 y 196** (Figura 5.9.) muestran como tanto a N/P 5 como N/P 10 son capaces de complejar y proteger con alta eficacia el material génico.

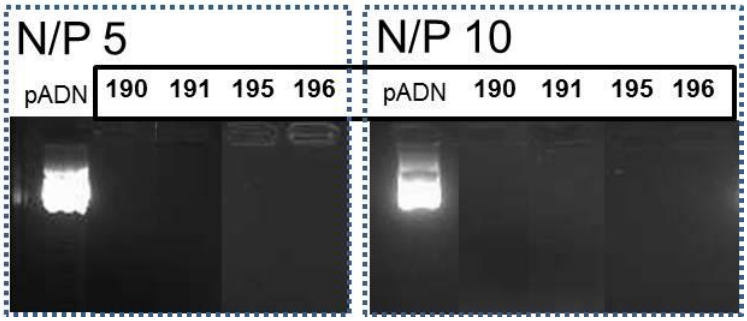


Figura 5.9. Electroforesis en gel de agarosa de las formulaciones de pADN y las ciclotrehalanas anfifílicas policatiónicas **190, 191, 195 y 196 a** valores de N/P 5 y 10.

Con objeto de confirmar que el pADN mantiene su integridad cuando está complejado por las ciclotrehalanas policatiónicas anfifílicas, los correspondientes complejos preparados a N/P 5 y N/P 10 se trataron con nucleasas (1U/μg ADN) durante 30 min a 37 ºC. Posteriormente, se inactivó la enzima y se añadió dodecilsulfato de sodio (SDS) para liberar el ADN complejado y visualizarlo mediante electroforesis en gel de agarosa (Figura 5.10.). Los geles indicaron que mientras que el pADN libre se degrada completamente bajo la acción de las nucleasas, los complejos con ciclotrehalanas lo protegen de manera muy eficaz.

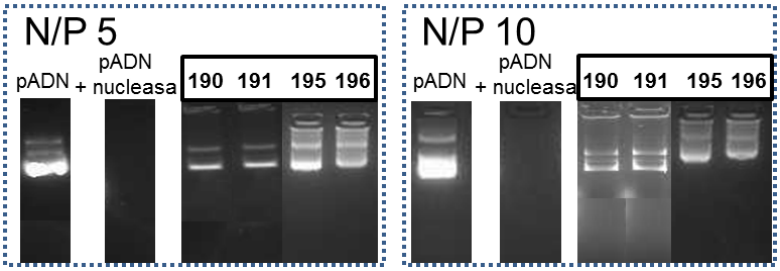


Figura 5.10. Ensayos de protección de ADN frente a nucleasas mediante experimentos de electroforesis en gel de agarosa de los complejos pADN-**190 (191, 195** y **196)**, a N/P 5 y N/P 10.

Determinación del diámetro hidrodinámico y el potencial ζ de los complejos con ADN mediante dispersión dinámica de la luz (DLS).

Un factor que dificulta la internalización del pADN libre a través de la membrana celular es su tamaño y carga negativa. La aplicabilidad de los vectores sintéticos depende de su capacidad de compactación y compensación de la carga negativa de los grupos fosfato. Para evaluar estas propiedades, se llevaron a cabo en primer lugar medidas de tamaño y carga de los complejos obtenidos a partir de los derivados anfifílicos de glucosa y trehalosa con pADN a valores de N/P 5 y 10 mediante dispersión dinámica de la luz (DLS) y la técnica de microelectroforesis combinada con laser doppler integrada en el propio equipo. Los datos se recogen en la Tabla 5.2.

Tabla 5.2. Diámetros hidrodinámicos (nm), potenciales ζ (mV) e índices de polidispersidad de los complejos formulados con los derivados anfifílicos de glucosa/trehalosa y pADN a N/P 5 y 10.

Comp	Tamaño N/P 5	Potencial ζ N/P 5	PI	Tamaño N/P 10	Potencial ζ N/P 10	PI
11	110.5±1.4	34.9±0.2	0.215	213.3±59.9	40.5±0.6	0.462
16	76.9±0.8	54.0±5.6	0.188	73.1 ± 2.80	62.1±4.7	0.214
12	416.8±186.8	25.6±0.8	0.685	96.6±4.4	36.7±2.5	0.222
52	3566.0±556.8	11.0±0.7	0.424	339.0±106.1	59.8±5.2	0.397
56	144.7±2.4	64.8±8.7	0.303	87.6±0.4	94.9±3.2	0.311
54	3438.0±533.9	-4.5±6.9	0.259	4342.0±169.6	3.6±2.4	0.317
58	2448±382.7	5.5±2.5	0.500	n.d.*	n.d.	n.d.
70	88.5±1.9	73.6±7.0	0.180	95.1±3.6	68.2±4.1	0.208
13	95.6±4.1	69.2±4.5	0.163	82.6±4.5	72.4±2.8	0.176
103	89.6±2.5	43.9±3.2	0.238	81.6±1.8	49.9±3.7	0.305
105	1334.0±1083.0	15.0±1.7	0.808	925±121.7	3.63±0.2	0.436
109	112.3±1.7	69.6±3.9	0.171	118.6±0.9	71.9±1.5	0.145
107	343.7±11.2	32.9±2.2	0.402	104.4±2.6	41.0±4.5	0.395

111	66.3±0.7	26.5±6.4	0.162	73.3±0.3	55.1±3.4	0.166

*Los experimentos de DLS de los complejos no proporcionaron sistemas nanoparticulares (n.d.: no determinados).

En lo que se refiere a los derivados de trehalosa, excepto **105** todos compactan de manera eficaz el ADN en este ensayo, dando lugar a partículas de tamaño nanométrico y con bajos índices de polidispersidad (PI). En el caso de los derivados de glucosa los índices de polidispersidad son mayores, con desviaciones estándar que en muchos casos son muy altas, lo que sugiere menor eficacia para la compactación del ADN. En general los diámetros hidrodinámicos disminuyen al pasar de N/P 5 a N/P 10.

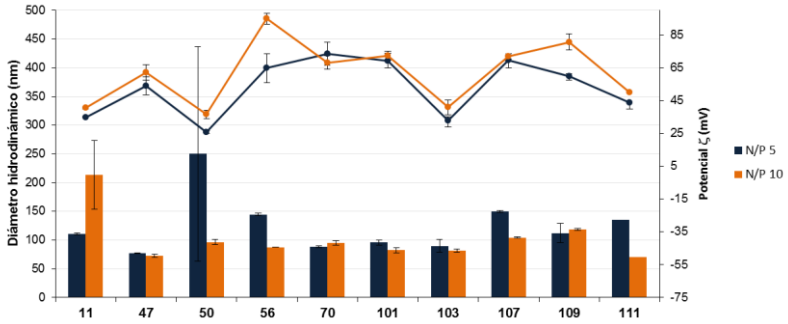


Figura 5.11. Diámetros hidrodinámicos (nm) y potencial ζ (mV) de los complejos formulados con **11, 47,50, 56, 70, 101, 103, 109, 107** y **111** y pADN a N/P 5 y 10.

Los valores de potencial ζ, determinados por M3-PALS (*Mixed Mode Measurement-Phase Analysis Light Scattering*), (Tabla 5.2, Figura 5.12) indican que los valores de carga superficial son algo más bajos en los derivados de glucosa que en los derivados de trehalosa, observándose un complejo con carga superficial negativa en el caso del derivado **54** para una relación N/P 5.

Los valores de diámetro hidrodinámico y carga superficial en el caso de **105** confirman su comportamiento poco eficaz para proteger el ADN, observado en electroforesis en gel de agarosa. Sin embargo, algunos derivados presentan valores de

tamaño y carga superficial aparentemente incoherentes con los resultados obtenidos en los experimentos de electroforesis. Por ejemplo, los compuestos **11** y **70**, mostraron valores de diámetro hidrodinámico bajos (110.5 y 88.5 nm) y de carga superficial altos (34.9 y 73.6 mV) a N/P 5 compatible con una compactación eficiente del ADN a bajas concentraciones. Por otra parte, el derivado **54** que mostraba gran eficiencia en la protección del ADN en gel de agarosa a N/P 5 y 10, presenta valores muy altos de diámetro hidrodinámico (3438 y 4342 nm) (Figura 5.11) y bajos de potencial ζ (-4.5 y 3.6 mV) (Figura 5.12), lo que sugiere una compactación del ADN en agregados microparticulares que no serían adecuados para terapia génica.

Los resultados de los experimentos de DLS para los complejos de ciclotrehalanas anfifílicas policatiónicas **190**, **195**, **191** y **196** con ADN se muestran en la Tabla 5.3. En todos los casos se observa que poseen tamaños nanométricos, cargas superficiales positivas (25-30 mV) y bajas polidispersidades. Los valores obtenidos son de hecho muy próximos a los obtenidos para ciclodextrinas policatiónicas anfifílicas como **ADM70**[14] que ha demostrado ser un excelente vector de transfección. Las diferencias de los diámetros hidrodinámicos y los valores de potencial ζ no son muy acusadas al pasar de N/P 5 a 10, lo que indica que ya a N/P 5 el ADN compactado y protegido del medio externo tal y como sugerían los experimentos de electroforesis.

Tabla 5.3. Diámetros hidrodinámicos (nm), potenciales ζ (mV) e índices de polidispersidad de los complejos de ADN formulados con las ciclotrehalanas policatiónicas anfifílicas a N/P 5 y 10.

Comp.	Tamaño N/P 5	Potencial ζ N/P 5	PI	Tamaño N/P 10	Potencial ζ N/P 10	PI
190	230.0±21.0	18.0±2.8	0.30	140.0±3.5	29.0±7.1	0.28
191	123.0±30.0	27.0±1.4	0.28	130.0±11.3	30.0±0.7	0.28
195	83.4±1.2	16.7±0.8	0.27	97.9±0.6	23.9±1.0	0.24
196	125.2±0.8	18.0±0.7	0.24	78.8±0.9	25.1±0.2	0.19

5.2.4. Evaluación de la eficiencia de transfección en células COS-7 y HepG2.

La eficacia de transfección de los complejos con pADN de los derivados **11**, **16**, **12**, **70**, **13**, **17**, **30**, **32**, **34**, **36**, **190**, **195**, **191**, **196** se evaluó en primer lugar, en células COS-7 (células de riñón de primate) usando el gen reportero de luciferasas eFLuc (pCpG-hCMV-SPEC-eLuc, 4640 pares de bases). Como control positivo se empleó el vector polimérico policatiónico comercial polietilenimina ramificada (bPEI, 25 kDa),. Los ensayos de transfección y viabilidad celular con los derivados de metil α-D-glucpiranósido y α,α'-trehalosa se realizaron en colaboración con el profesor Christophe Di Giorgio, de la Universidad de Niza, mientras que los experimentos con los derivados de ciclotrehalanas se llevaron a cabo en colaboración con la profesora Concepción Tros de Ilarduya

Los niveles de luciferasa expresada, indicativos de la cantidad de ADN transfectado, se determinaron por medidas de luminiscencia tras añadir el sustrato de la enzima (luciferina). La viabilidad celular se midió, en el caso de los complejos de los derivados de glucosa y trehalosa, a partir de la relación entre la cantidad de proteínas producidas por las células transfectadas y por células control no tratadas con los complejos. En el caso de las ciclotrehalanas, se utilizó el ensayo alamarBlue (*Invitrogen*). Este ensayo aprovecha el alto poder reductor de las células vivas para cuantificar la supervivencia de las células y establecer la viabilidad celular durante la transfección. El reactivo utilizado es azul en su forma oxidada y permite determinar las células vivas ya que al entrar en contacto con el citoplasma de éstas pasa a su forma reducida de color rojo fluorescente.

Los primeros ensayos de transfección y viabilidad celular se llevaron a cabo en ausencia de suero (suero fetal bovino, FBS) con los derivados de metil glucosa **11**, **16**, **12** y de trehalosa **70**, **13**, **17** (Figura 5.13.) utilizando como control positivo la polietilénimina (PEI), un vector empleado como referencia en transferencia no viral, y el pADN desnudo como control negativo.

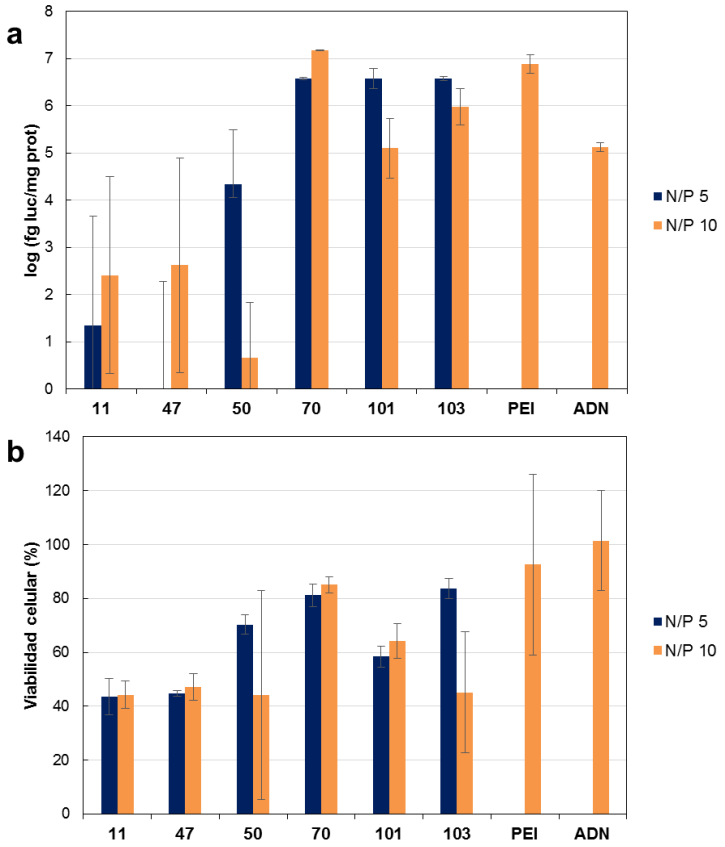


Figura 5.13. Viabilidad celular (a) y eficacia de transfección (b) de los complejos de ADN formulados con PEI y los derivados **11**, **47**, **50, 70**, **101**, y **103** a N/P 5 (barras azules) y N/P 10 (barras naranjas) en células COS-7.

Los datos recogidos en la Figura 5.13a indican que los derivados de metil glucósido **11**, **16**, **12** muestran una toxicidad mayor que los análogos de trehalosa **70**, **13**, **17**, sobre todo a valores de N/P 5. Cabe destacar la viabilidad celular del 85% en el caso del derivado **70** a N/P10. Los derivados de metil glucósido mostraron niveles de transfección prácticamente nulos (Figura 5.13b) mientras que los derivados de trehalosa muestran capacidades de transfección similares o superiores al PEI.

A la vista de estos resultados, se llevaron a cabo ensayos de viabilidad celular y transfección de los derivados de trehalosa **70**, **13**, **17**, **30**, **32**, **34**, **36** en presencia de un 10% de suero (FBS), con objeto de evaluar su potencial en aplicaciones sistémicas. En general, la toxicidad de los complejos de trehalosa a valores de N/P 5 es inferior a la del PEI a N/P 10 (condiciones óptimas para este vector; 46% de viabilidad celular) Sin embargo a relaciones N/P 10) se produce un aumento drástico de la mortalidad celular en el caso de los derivados de trehalosa **70**, **13**, **17** (Figura 5.14a). Por el contrario los complejos formulados con los derivados **105**, **32**, **34**, **36**, que incorporan el anillo de triazol en su estructura, muestran viabilidades celulares mucho mayores, claramente superiores a la del PEI, tanto a valores de N/P 5 como 10 (Figura 5.14a). El efecto positivo de los derivados de triazol en medios con presencia de suero ya había sido observado en complejos de ciclodextrinas policatiónicas anfifílicas con ADN..[20]

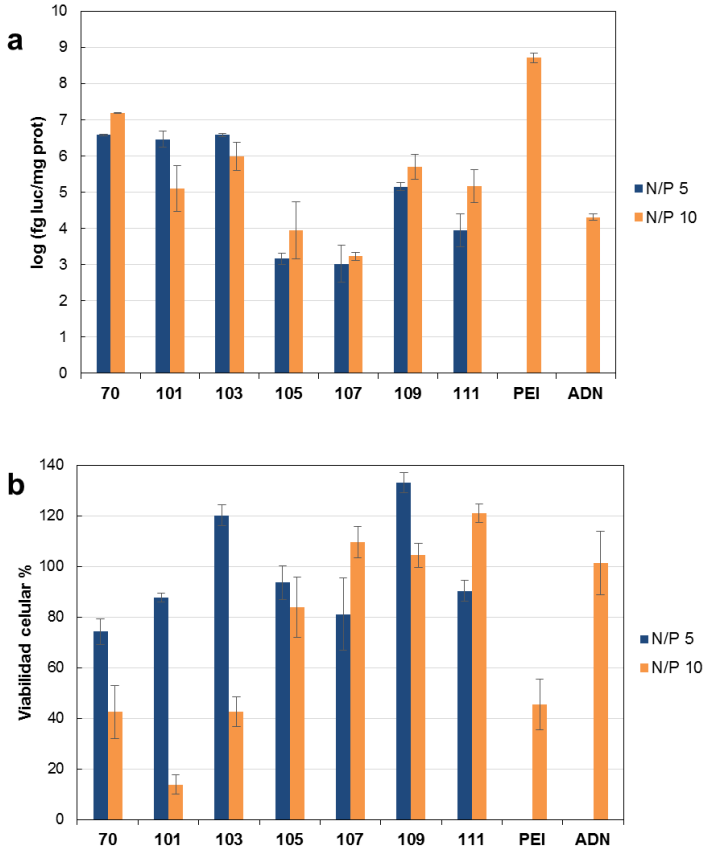


Figura 5.14. Viabilidad celular (a) y eficacia de transfección (b) en células COS-7 de los complejos de ADN formulados con PEI, y de trehalosa **70**, **101**, **103, 105, 109, 107** y **111** a N/P 5 (barras azules) y N/P 10 (barras naranjas) en presencia de un 10% de FBS.

En los ensayos de transfección en presencia de suero (Figura 5.14b), los niveles de expresión de luciferasa fueron siempre inferiores a los del PEI (Figura 5.13b). Los derivados **70**, **101**, **103**, a pesar de presentar peores viablilidades celulares, muestran una expresión de luciferasa superior a la de los derivados que incorporan anillos de triazol en su estructura (**104, 109, 107 y 111**). No obstante este efecto puede deberse a que la

elevada mortalidad celular en los derivados sin anillo de triazol **70**, **101**, **103** produce una sobreestimación de la expresión normalizada de luciferasa.

Los ensayos de viabilidad celular y transfección en célulasCOS-7 con los complejos de las ciclotrehalanas **190**, **191**, **195** y **196** y pADN (CTplejos) se llevaron a cabo a relaciones N/P 5 y N/P 10 en ausencia y presencia de suero (10% y 60% de FBS) Los resultados obtenidos (Figura 15.5) muestran una toxicidad muy baja para todos los CTplejos independientemente del valor de N/P y de la concentración de suero (Figura 5.15), con valores de supervivencia celular superiores al PEI. No se observan diferencias significativas entre los derivados hexanoilados (**190**, **195**) y miristoilados (**191**, **196**) en lo que se refiere a la toxicidad de los complejos.

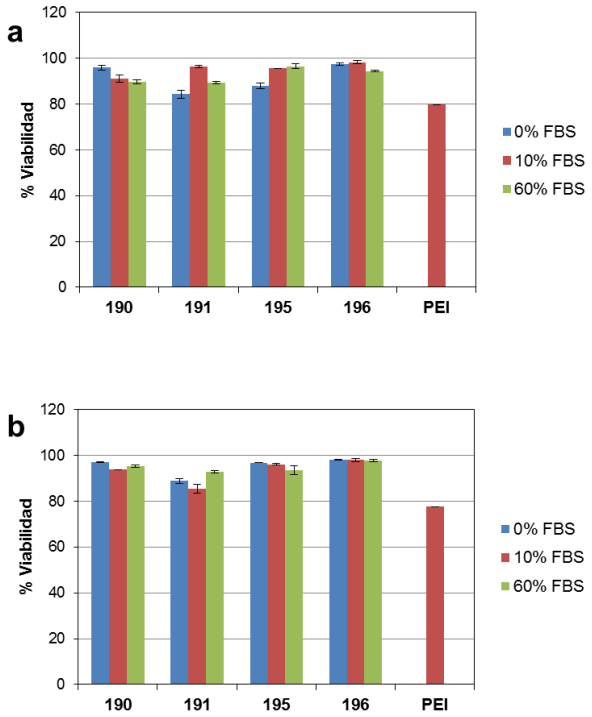


Figura 5.15. Viabilidad celular a N/P 5 (a) y N/P10 (b) en células COS-7 de los complejos de ADN formulados con PEI y los derivados de ciclotrehalanas **190**, **195**, **191 y 196** en ausencia de suero (barras azules) y con proporciones de FBS al 10% (barras rojas) y 60% (barras verdes).

Contrariamente a lo observado para la viabilidad celular, los resultados de transfección (Figura 5.16) indican una influencia decisiva del balance entre grupos amonio y cadenas grasas. El aumento de hidrofobicidad al sustituir las cadenas de hexanoilo por cadenas de miristoilo influye negativamente en la expresión de luciferasa de los derivados **191** y **196**. Los derivados hexanoilados **190** y **195** muestran eficacias de transfección superiores al PEI incluso en presencia de un 60% de suero y a valores de N/P 5. Al aumentar la relación N/P de 5 a 10 la eficacia de transfección disminuye en presencia de suero de manera mucho más marcada, lo que sugiere que el aumento de la concentración del vector puede inducir fenómenos indeseados de agregación con las proteínas del suero. En el caso de los derivados miristoilados **191**, **196**, aunque el aumento del valor de N/P produce un incremento significativo en la capacidad de transfección, en presencia de un 60% de suero se produce una disminución casi total de los niveles de expresión de luciferasa.

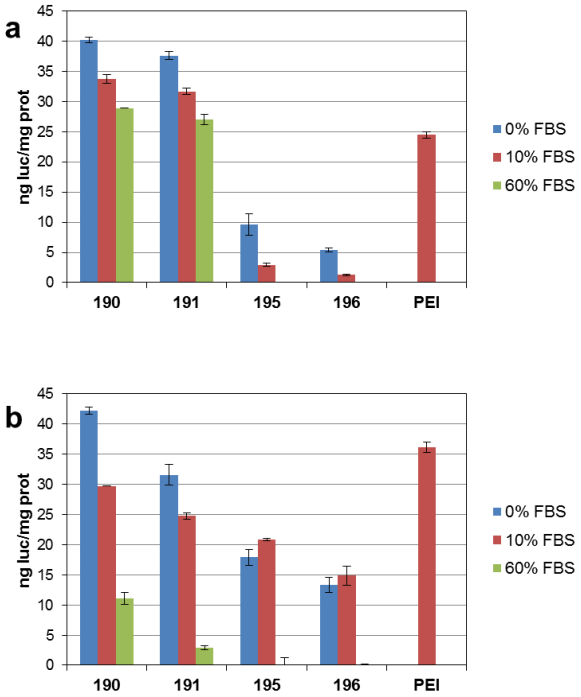


Figura 5.16. Eficacia de transfección celular a N/P 5 (a) y N/P10 (b) en células COS-7 de los complejos de ADN formulados con PEI y los derivados de ciclotrehalanas **190**, **191**, **195** y **196** en ausencia de suero (barras azules) y con proporciones de FBS al 10% (barras rojas) y 60% (barras verdes).

A la vista de los buenos resultados obtenidos en las células COS-7, los derivados de CTs se evaluaron adicionalmente en una línea celular de relevancia médica, las células HepG2 de hepatoblastoma humano, con el fin de evaluar el potencial de estos nuevos vectores en terapias contra el cáncer. Los ensayos se llevaron a cabo utilizando el mismo procedimiento que en células COS-7 (pADN eFLuc, N/P 5 y 10, en ausencia y en presencia de suero al 10 y al 60%). Todos los derivados policatiónicos de ciclotrehalanas mostraron niveles de viabilidad celular superiores al 95% bajo todas las condiciones estudiadas de valor N/P y porcentaje de FBS (Figura 5.17), por encima del valor del agente de transfección comercial de referencia PEI, como sucedía en las células COS-7.

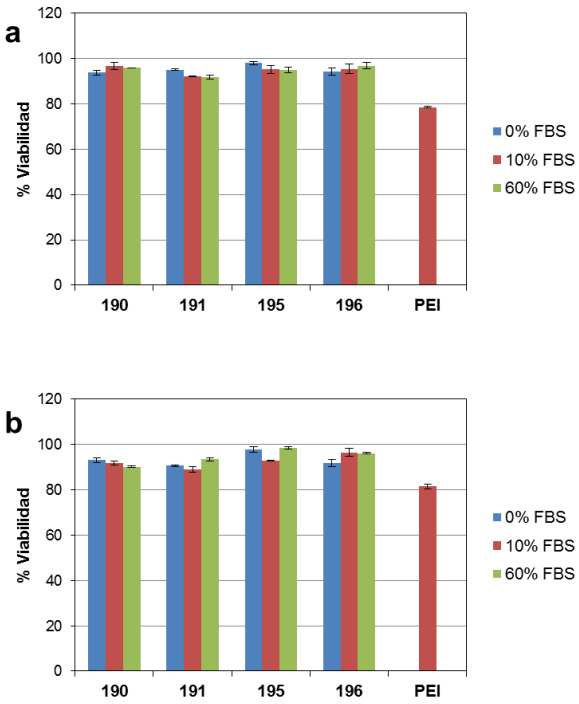


Figura 5.17. Viabilidad celular a N/P 5 (a) y N/P10 (b) en células HepG2 de los complejos de ADN formulados con PEI y los derivados de ciclotrehalanas **190**, **191**, **195** y **196** en ausencia de suero (barras azules) y con proporciones de FBS al 10% (barras rojas) y 60% (barras verdes).

Los resultados de eficacia de transfección en células HepG2 para los CTplejos son similares a los obtenidos en células COS-7. El derivado **190** muestra niveles de expresión superiores al PEI a valores de N/P 5 en presencia de 10% de FBS. Aunque la eficacia de transfección disminuye al aumentar la proporción de suero, en presencia de 60% de FBS todavía es comparable a la del PEI con sólo el 10% de suero (Figura 5.18). La eficiencia de transfección de los derivados miristoilados **191**, **196** fue inferior en todos los casos a la de los análogos hexanoilados.

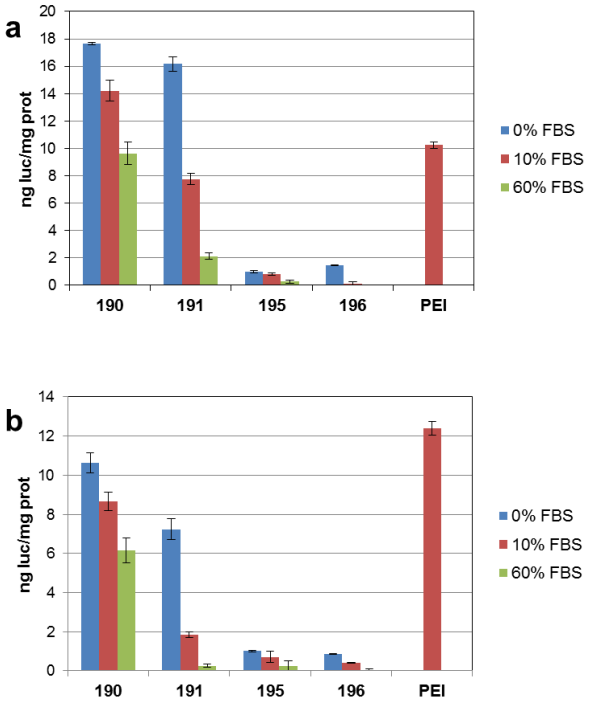


Figura 5.18. Eficacia de transfección celular a N/P 5 (a) y N/P10 (b) en células HepG2 de los complejos de ADN formulados con PEI y los derivados de ciclotrehalanas **190**, **191**, **195** y **196** en ausencia de suero (barras azules) y con proporciones de FBS al 10% (barras rojas) y 60% (barras verdes).

Por último, se han llevado a cabo ensayos de transfección y expresión in vitro con el gen terapéutico que codifica la interleuquina-12 (IL12), empleando los CTplejos formulados con las CTs **190**, **191**, **195** y **196** y el plásmido pCMV-IL12, en células HepG2 a valores de N/P 5 y 10, y en ausencia y presencia de suero (10% y 60% de FBS). Se observó una eficacia de transfección muy superior para el derivado **190** (Tabla 5.3.) con valores de expresión de IL-12 superiores a los del agente de transfección de referencia PEI. La adición de suero no afecta significativamente la transfección en el caso de las ciclotrehalanas. En resumen, los resultados obtenidos indican que el derivado de ciclotrehalana **190** es un buen candidato para estudiar su capacidad de transfección y

expresión génica *in vivo*. Actualmente este tipo de experimentos se están llevando a cabo en el laboratorio de la Profesora Tros de Ilarduya.

Tabla 5.4. Eficacia de transfección celular a N/P 5 y N/P10 en células HepG2 de los complejos de ADN IL-12 formulados con PEI y **190**, en ausencia de suero presencia de FBS al (0, 10, 60%).

Comp.	% FBS	N/P 5	N/P 10
190	0	3.19 ± 0.33	2.12 ± 0.53
	10	4.57 ± 0.29	1.68 ± 0.29
	60	2.90 ± 0.65	1.65 ± 0.44
bPEI	10	-	0.02 ± 0.01

En este capítulo se han presentado los resultados obtenidos para una serie de derivados (poli)catiónicos anfifílicos basados en metil α-D-glucopiranósido y α,α'-trehalosa en lo que se refiere a sus propiedades de agregación y su capacidad para complejar pADN y promover la transfección. En el estudio también se han incluido otros vectores de transfección sintéticos basados en dímeros cíclicos de α,α'-trehalosa que incorporan cadenas grasas y grupos amino protonables en regiones opuestas de la estructura (ciclotrehalanas policatiónicas anfifílicas; paCTs). La homogeneidad de todos los compuestos se ha demostrado mediante espectroscopia de FTIR, RMN, espectrometría de masas y análisis elemental. Los compuestos anfifílicos mono- y disacarídicos forman agregados nanométricos con cargas superficiales positivas en ausencia y presencia de ADN, pero mientras que en el primer caso la polidispersidad es grande, en presencia del oligonucleótido el ordenamiento es mucho más regular. Lo mismo sucede con los derivados de ciclotrehalanas. Los experimentos de electroforesis confirmaron la capacidad de compactación del ADN de estos derivados anfifílicos, dando lugar a complejos en los que el ADN está protegido frente a la acción de nucleasas. No obstante, las paCTs demostraron ser más efectivas en la protección del ADN que los derivados mono o disacarídicos. Los ensayos de transfección con vectores basados en metil α-glucopiranósido indicaron una baja eficacia. Los derivados de trehalosa fueron mucho

más eficaces en ausencia de suero, pero dieron lugar a toxicidades elevadas en presencia de un 10% de suero. Por el contrario, los complejos formulados con los derivados de CTs, CTplejos, mostraron viabilidades celulares elevadas incluso en presencia de altos porcentajes de suero (60%, viabilidad celular ≥ 95%). Los ensayos de transfección en células COS-7 y HepG2, utilizando un gen reportero codificante de luciferasa, a valores de N/P 5 y 10 indicaron que los derivados de ciclotrehalanas con cadenas de hexanoilo son mucho más eficaces que los derivados que incorporan cadenas grasas de miristoilo. Finalmente, se llevaron a cabo ensayos de transfección en células HepG2 utilizando un plásmido terapéutico que codifica la interleuquina-12 (IL-12), observándose que incluso en presencia de altos porcentajes de suero y a valores de N/P 5, el nanpcomplejo formulado con la paCT **190** presenta niveles de transfección similares al vector de referencia PEI pero con mejores viabilidades celulares. Estas excelentes propiedades como transportadores de genes de las paCTs, comparables a la de las ciclodextrinas policatiónicas anfifílicas (paCDs), hacen de estos derivados potenciales candidatos para su aplicación en ensayos *in vivo* en terapias contra el cáncer. Estos experimentos se están realizando actualmente en colaboración con la Profesora Tros de Ilarduya y, simultáneamente, se están preparando en nuestro grupo nuevas series de paCTs que permitan optimizar sus propiedades como vectores de transfección.

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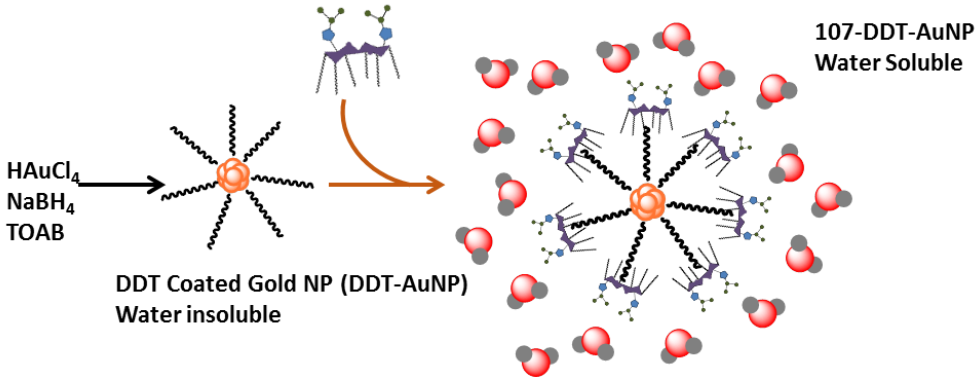
Chapter 6

Trehalose- and glucose- derived glycoamphiphiles: small-molecule and nanoparticle Toll-Like receptor 4 (TLR4) modulators.

Abstract: Toll-like receptors (TLRs) are a class of transmembrane protein receptors involved in the early recognition of pathogens, playing a pivotal role in the innate immune system. TLR4 specifically recognizes lipopolysaccharide (LPS) of gram-negative bacteria, and its misregulation is involved in a wide range of disorders, of which septic shock constitute the major life threatening syndrome. For such problem, TLR4 is now an emerging molecular target related not only to septic shock, but also to an impressively broad spectrum of modern day disorders.

We present a complete evaluation of the glycoderivatives presented in chapter 4 as TLR4 antagonists, including the evaluation of the best candidate into a multivalent presentation based on gold nanoparticles.



6. Trehalose- and glucose-derived glycoamphiphiles: small-molecule and nanoparticle Toll-Like receptor 4 (TLR4) Modulators.

6.1. Introduction

Toll-like receptors (TLRs) are a class of protein that play a pivotal role in the innate immune system. They're single, membrane-spanning, non-enzymatic receptors usually expressed in macrophages and dendritic cells. They're named TLRs because of their similarity to the Toll gene identified in *Drosophila melanogaster*. Thus, TLRs are a class of pattern recognition receptors (PRR). They recognize structurally conserved pathogen-associated molecular patterns (PAMPs), molecules broadly shared by pathogens such as viruses, protozoa, fungi and bacteria and triggers the innate immune response.[1] TLRs together with the interleukin-1 (IL-1) receptors form a receptor superfamily, known as ''Interleukin-1 Receptor/Toll-Like Receptor Superfamily''.[2] TLRs are present in vertebrates, as well as invertebrates. Some genes, with similar sequence to TLRs have been also found in many plants[3] and seem to be required for host defense against infections. TLRs seems to be one of the most widespread components of the animal immune system, most mammalian species having between ten and fifteen types of TLRs identified so far. TLR1 to TLR13 have been found in humans and mice, and other forms have been found in other mammalian species[4,5,6] TLRs bind specifically to molecules that are constantly associated with threats (i.e. pathogens or cell stress) and are highly specific to these threats. These features in microbes include bacterial lipopolysaccharide (LPS), lipoproteins, lipopeptides, double-stranded RNA from viruses, viral DNA (Figure 6.1.). It has been proposed that endogenous activators of TLRs might participate in autoimmune disorders.[7] TLRs are thus activated by different ligands[5], which when activated, recruit adapter molecules within the cytoplasm of cells in order to propagate a signal. At least four molecules (proteins) are known to be involved in signaling: MyD88, TIRAP, TRIF and TRAM.[8] This adapter molecules activate others protein kinases that amplify the signal, and, finally, causes the activation or suppression of genes that orchestrate the inflammatory response.

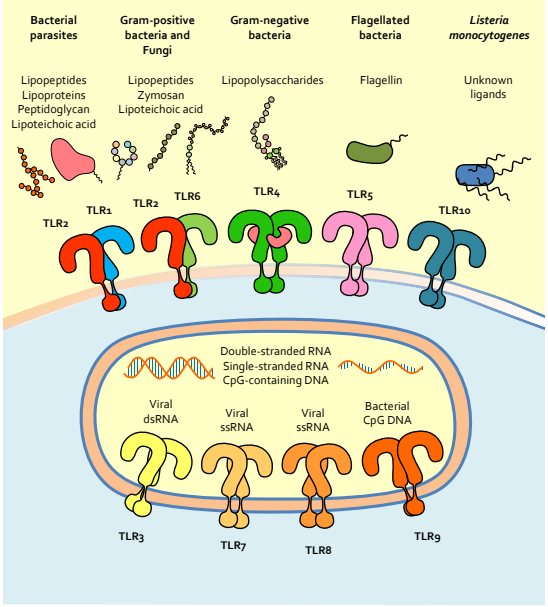


Figure 6.1. Location and targets of some TLRs. TLRs are present either on cell membranes or in intracellular compartments such as endosome.

TLR4 is a cell surface, single-spanning transmembrane protein containing 799 aminoacid residues, with an extracellular leucine-rich repeat (LRR) domain and an intracellular TIR domain (Figure 6.2.) Among TLR's, TLR4 selectively recognizes lipopolysaccharides (LPS), also known as endotoxin (E). LPS is the main component of Gram negative bacteria outer membrane, being the Lipid A, the hydrophobic part of LPS, the bioactive moiety, responsible for its proinflammatory activity.[9] Lipid A is a negatively charged, phosphorylated lipodisaccharide. Lipid A is slightly structurally different depending on the different bacterial origin, exhibiting a variety of chemical modifications mainly involving the lipophilic chains. Some structural components have been recognized to be essential for the endotoxic activity, such as the presence of a β(1-6) linked diglucosamine backbone, diphosphorylation at the anomeric C-1 and C-4' position, and a suitable number and location of three acyl chains per disaccharide unit (Figure 6.2.) The induction of inflammatory process by LPS initiates the production of multiple endogenous mediators, including cytokines (e.g. tumor necrosis factor, TNFα), arachidonic acid metabolites and tissue factors. TLR4 does not bind directly to LPS: the molecular pathway is a complex process involving a sequential action of four principal proteins, starting with the LPS-binding protein (LBP)-catalyzed extraction and transfer of individual LPS molecules from its aggregates in form of micelles or membrane blebs[10], to the CD14 (cluster of differentiation-14) receptor[11] The role of CD-14 seems to be fundamental when endotoxin has very low concentration, while in high amounts TLR4 can by efficiently stimulated by LPS in absence of CD14. Then, the LPS monomers are conducted from CD14 to MD-2 protein (myeloid differentiation protein 2)[12,13]. This process is followed by engagement

and dimerization of TLR4 forming the supramolecular dimmer $(LPS{\cdot}MD\text{-}2{\cdot}TLR4)_2$[14] which triggers the intracellular signaling activating the downstream pathways (Figure 6.3.). TLR4 is also activated by endogenous factors, generally knows as danger associated molecular patterns (DAMPs)[15] released as a consequence of injury or inflammation, including β-defensin, hyaluronic acid, heparin sulfate, substance P and many others.

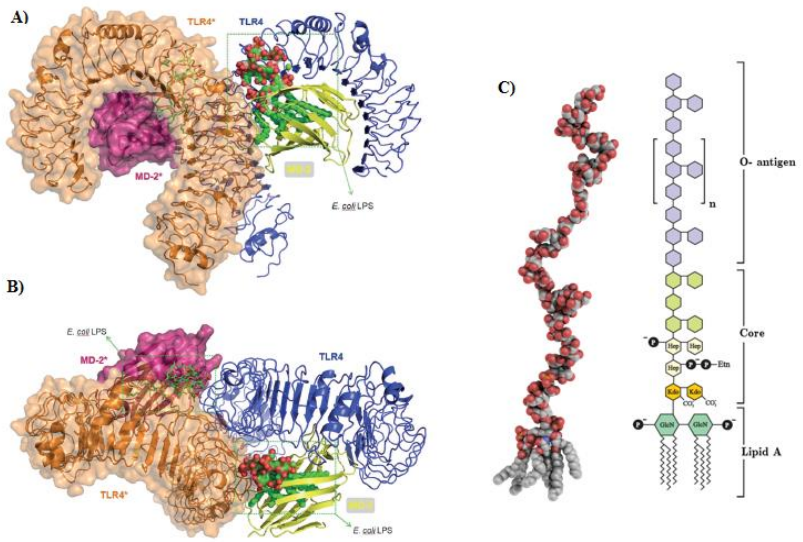


Figure 6.2. X-ray structure of the TLR4/MD-2/LPS complex (PDB ID 3FXI). A) Front view. B) Top view. C) Generic structure of Lipopolisaccharide. The O-antigen varies between different species of gram-negative bacteria, while Lipid A is a conserved structure, although some modifications of its structure have been reported among bacteria.

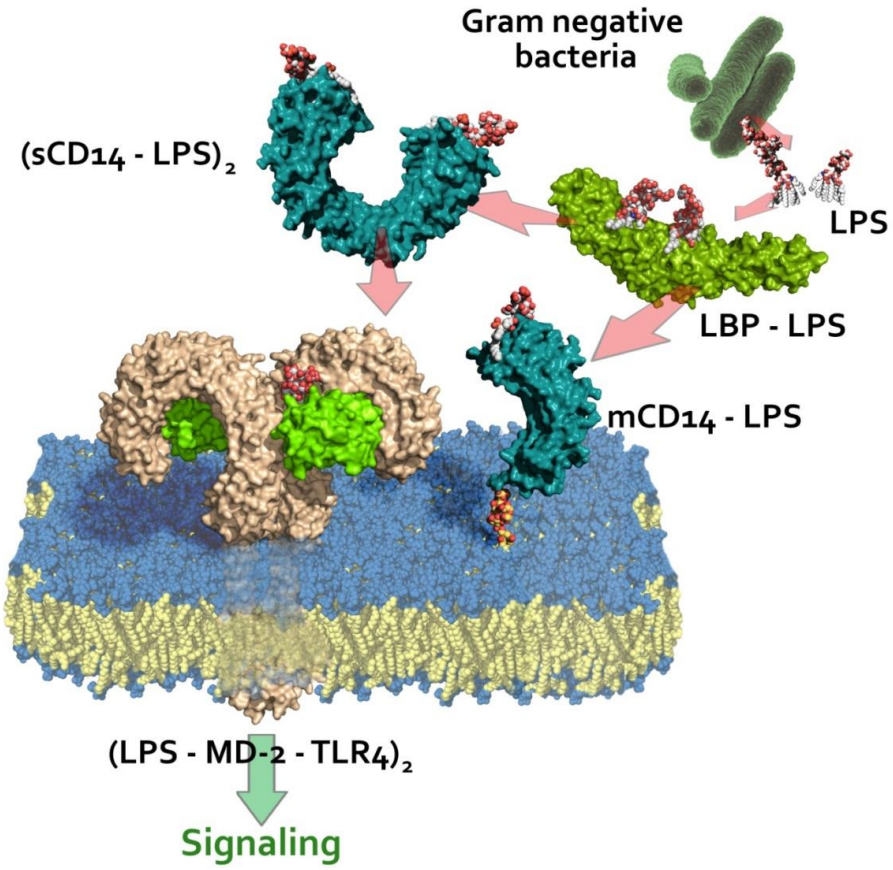


Figure 6.3. TLR4–associated proteins that are involved in LPS sensing. Accordingly to the currently accepted mechanism, the first protein involved in the process is the LBP, then LPS is bound CD14 (indicated as sCD14 (soluble form) or mCD14 (membrane anchored form)) and finally, it interacts with MD-2 protein, which self-associate with TLR4 receptor, inducing its dimerization and triggering the inflammatory response.

Excessively potent and deregulated TLR4 activation and signaling causes severe syndromes such as septic shock[16], associated with a high mortality (50–70%), and organ-specific damages. According to literature, TLR4 is an emerging molecular target related to an impressively broad spectrum of modern day disorders, such as asthma[17], cardiovascular disorder, diabetes, obesity, metabolic syndrome, autoimmune disorders, neuroinflammatory diseases, neurophatic pain[18], Central Nervous System diseases like Amyotrophic Lateral Sclerosis (ALS) or Alzheimer disease, psychiatric diseases, dermatitis, psoriasis and some tumors. The majority of the mentioned pathologies lack specific treatment, so that TLR4 modulation by small molecules has attracted increasing interest. Efficient and selective TLR4 antagonists with chemical structures simpler than that of lipid A are therefore required for the development of potential new drugs with a wide array of medical and pharmacological applications.

Several natural, synthetic and semisynthetic compounds are known to modulate TLR4 pathway, acting either as agonist, that activate TLR4 in the same way of LPS does, or

antagonist, thus inhibiting TLR4 pathway by blocking the interaction in one of the possible levels (inhibiting LPS to be transferred to LBP, blocking the CD14 extraction of monomers, or inhibiting the interaction of LPS with MD-2·TLR4).[10] The majority of this modulators are natural or synthetic Lipid A variants that imitate the structure of the original motif of the Lipid A. In general, these variants are anionic glycolipids with negatively charged groups, such as phosphate or sulfate, bearing lipophilic chains, being the synthetic compound Eritoran one of the best known molecules. (Figure 6.4., eritoran). Although it may be counterintuitive, not only anionic glycolipids, but also cationic derivatives have been reported as active in modulating TLR4 pathway. Several molecules bearing positively charged groups (tertiary or quarternary amines mainly) an a hydrophobic motif (alkyl or acyl chains) have been found to modulate TLR4 activity[19] Also, positively charged liposomes made of self-assembling cationic amphiphilic compounds can induce the expression of proinflammatory mediators. DiC14-amidine (Figure 6.4,) liposomes have been shown to trigger the secretion of a cytokine pattern reminiscent of the TLR4-dependen LPS secretion pattern by activating both MyD88/NF-κB/JNK and TRAM/TRIF pathways.[20] Other cationic lipids activate cytokine production through NF-kB independent/TRIF-dependent pathways, with requires the presence of CD14[21,22]Subtle structural changes make some cationic lipids to switch from agonism to antagonism, as in the case of dioleoyl trimethylammonium propane (DOTAP), a cationic lipophilic molecule, mainly used as transfection agent,[23] that inhibits TLR4 pathway by competing with LPS for the interaction with LBP.[22] Interestingly DOTAP is not the only transfection agent found to modulate TLR4 pathway: LPS complexed with Lipofectamine® reduces its TLR4 agonism[24]

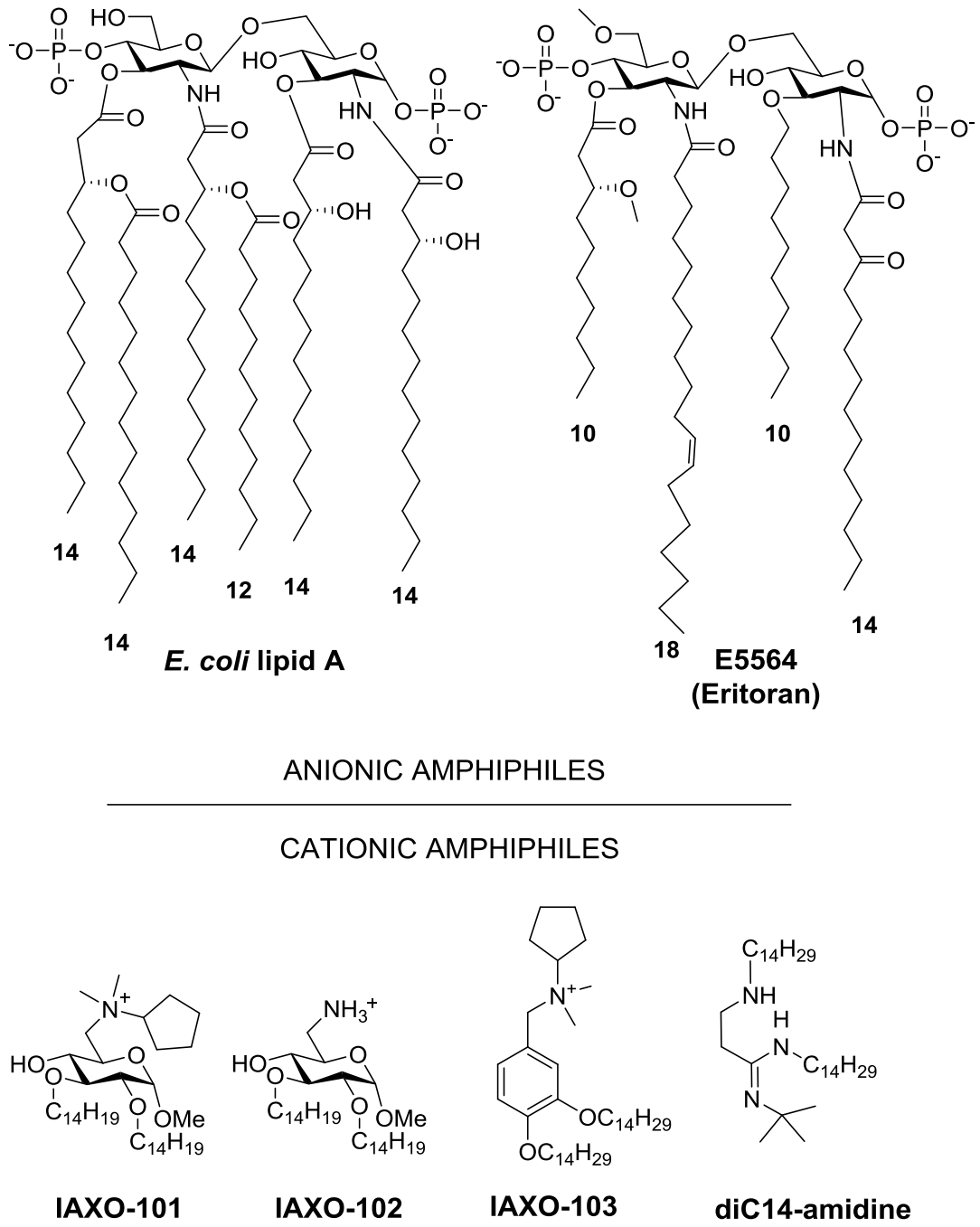


Figure 6.4. Anionic (up) and cationic (down) TLR4 modulators: Escherichia coli Lipid A (the natural TLR4 agonist, Eritoran (synthetic antagonist), IAXO-101, IAXO-102, IAXO-103 and diC14-amidine (cationic antagonists)..

The group of Prof. F. Peri (University of Milano-Bicocca) has a wide experience in this area, and has described many cationic lipids made of positively charged headgroups (amines) and lipophilic chains, having a carbohydrate or aromatic scaffold. Specific binding of this aminolipids to CD14 was recently shown by Prof. Peri, being active in inhibiting LPS-stimulated TLR-activation in vitro and in vivo.[25,26,27] The authors suggest that the lipid tails of the cationic amphiphiles interacts with the hydrophobic binding site of CD14, thus competing

with lipopolysaccharide for the active site.[28] Interestingly, through the interplay of regioselective functionalization methodologies and conformational bias, the installation of differentiated cationic and hydrophobic domains in carbohydrate platforms can be made compatible with molecular diversity-oriented strategies and structure-activity relationship (SAR) studies.

Due to the lack of structural data of cationic compounds bounding to CD14 or MD-2, very little is known on the molecular mechanisms behind the interaction. For such purpose, conducting SAR studies for this kind of compounds make possible relating the biological activity to the structure, thus helping to understand the process at molecular level. Glucose-derived cationic amphiphiles are particularlyattractive for these studies. The secondary hydroxyls of the glucopyranose unit are well suited anchoring points to link lipid chains in a very similar orientation as the lipid chains in lipid A. Also, the possibility of incorporation of protonable amino groups in the primary position confers amphiphilicity to the compound, improving its membrane crossing capabilities and allowing the formation of supramolecular aggregates such as micelles or nanoparticles. In the other hand, trehalose derivatives can be formally considered as dimeric analogues to the glucose counterparts with a better defined face differentiation between the cationic and the lipophilic domains.

Considering the availability of a large series of glusoe and trehalos (poly)cationinc anfifiles issued of this Thesis, and on the basis of the above considerations, we started a collaboration with the group of Prof. Peri culminating in a 4 months stay of the PhD candidate in the University of Milano-Biccoca. During this period, a complete set of ‘’skirt-like’’ trehalose and glucose based aminoglycolipids were assayed for their TLR4 modulating capabilities. It was known that the self-assembling behavior and the ability of cationic amphiphiles to interact with CD14 protein by forming liposomes or micelles hasa strong impact on their TLR4 modulatory activity.[19] Both scaffolds, methyl α-D-glucopyranoside and α,α’-trehalose have been previously used for elaboration of anionic anfifilic structures with TLR4 modulator capabilities.[29,30] Evaluation of cationinc analogues seemed thus interesting. Eventually, we could identify at least three new potentialdrug leads, one of which was suitable to be further incorporated in dodecanethiol-coated gold nanoparticles (AuDDT) in order to evaluate the effect of a multivalent presentation of the epitopes on its biological activity.

6.2. Results and Discussion

After a preliminary test, in which we evaluated the complete set of skirt-like glucose and trehalose derivatives, only the best candidates were evaluated in depth, while inactive molecules

or inconclusive molecules compounds affording inconclusive results were discarded. The molecules we finally decided to include in a full study are shown below (Figure 6.5.).

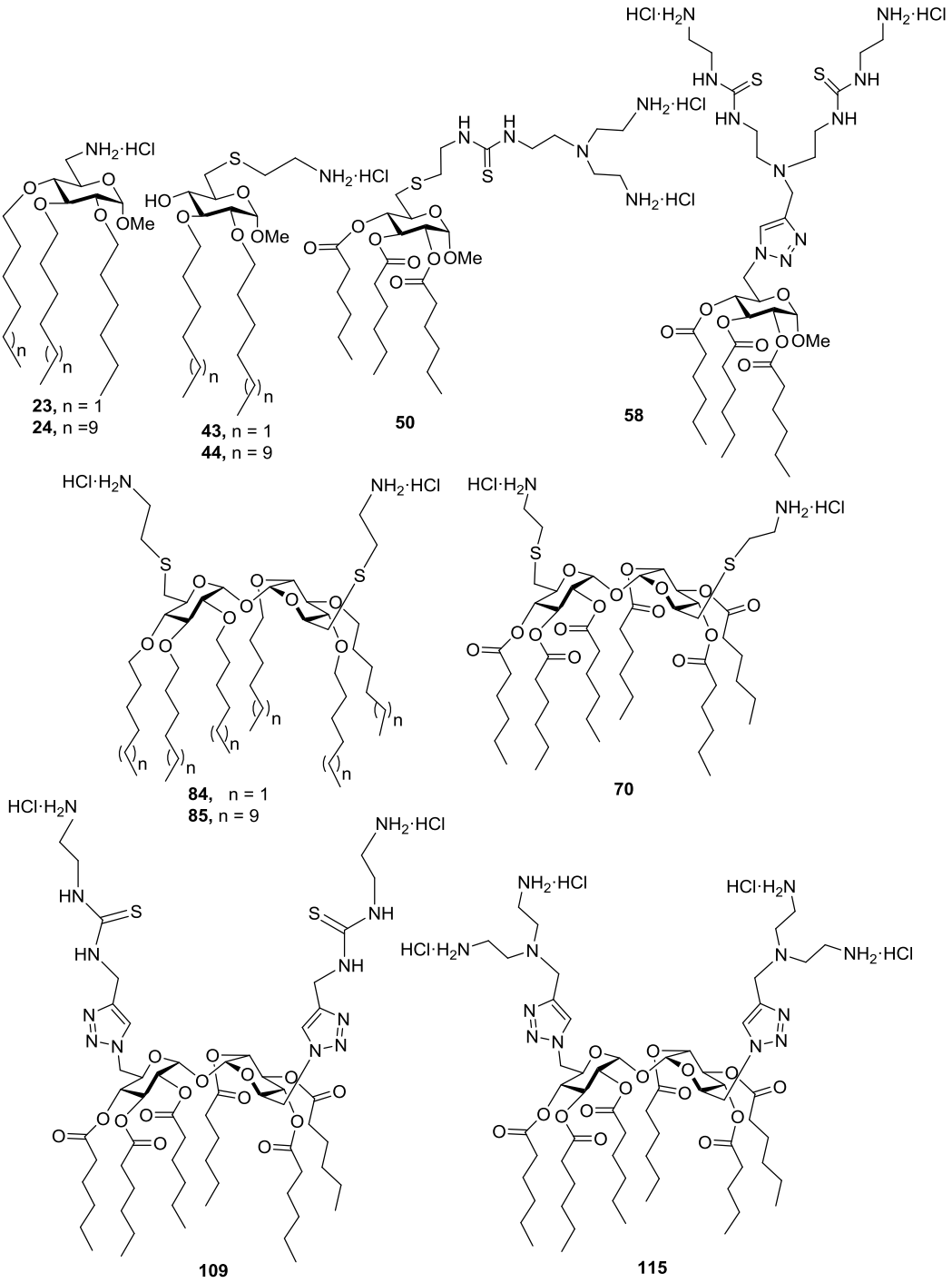


Figure 6.5. Structures of the compounds included in this study.

6.2.1. TLR4 modulation in HEK-Blue™ cells by cationic glycoamphiphiles.

At first, compounds X-Y were screened in order to evaluate their capacity to interfere with TLR4 activation and signaling in HEK-Blue™ cells. HEK-Blue cells arise from a Human Embrionic Kidney cell line stably transfected with TLR4, MD-2 and CD-14 genes and also, a

NF-κB-inducible SEAP (secreted embryonic alkaline phosphatase) reporter gene that can be conveniently monitored using the SEAP detection media.[29] Compounds **58**, **70**, **109**, **107** are inactive in stimulating TLR4 pathway when provided in the absence of LPS (no agonist activity) while inhibited, in a dose-dependent manner, the LPS-stimulated TLR4 signaling, thus showing antagonist activity (Table 6.1.). In the other hand, compounds **23**, **24**, **JB014**, **JB010**, **58**, **EMA07** and **EMA018** were inactive. Compounds having alkyl (ether-like) hexyl o tretradecyl chains resulted as being inactive or having a very low activity, acting as agonists or inhibitors. It seems to be a prerequisite for TLR4 antagonist activity the presence of acyl (ester-like) chains. For the acylated sublibrary, all compounds are active, except compound **58**, probing that the cationic head has also a pivotal role in TLR4 antagonist activity. Trehalose based compounds (**70**, **109**, **107**) showed higher potency than the active monosaccharide **50**. The results strongly point to a relationship between facial amphiphilicity and antagonist activity. Compound **107** showed the highest potency in TLR4 inhibition, in agreement with the suggested argument. All active compounds were further evaluated for their toxicity by MTT viability test, all of them being nontoxic (or exhibiting very low toxicity) in the concentration range studied (Figure 6.6).

6.2.2. TLR4 modulation in HEK-293 cells transfected with human (h) and hurine (m) MD2·TLR4.

Biologically active and nontoxic cationic glycolipids **50**, **70**, **109** and **107** were evaluated for their ability to interact with TLR4 pathway in HEK293 cells stably transfected with human or murine TLR4 and MD-2 genes, coupled to a dual luciferase reporter gene that allow the monitorization of the process. In the absence of LPS, none of the selected compounds were able to activate TLR4 signaling neither in cells with hMD-2·hTLR4 nor mMD2·mTLR4, so no agonist activity is present. On the contrary, when LPS is present, all molecules inhibited TLR4 pathway in a dose-dependent way, all of them having a similar potency in HEK293 transfected with human and mouse receptors, and very similar to the potency related for HEK-Blue™ cells (Figure 6.5, Table 6.1.). In HEK293 cells, **109** and **107** exhibited the highest potency both in mouse and human MD2·TLR4, being reminiscent of the activity of Eritoran[31, 32]that is a potent TLR4 antagonist in both species and contrasting with other compounds such as LipidIVa, which shows agonist activity in murine, but is a potent antagonist in human.[33]

Table 6.1. TLR4 antagonist activity of cationic glycolipids **50**, **70**, **109** and **107** on HEK-Blue Cells, HEK293 hMD- 2/hTLR4, and HEK293 mMD-2/mTLR4 stimulated with E. coli O55:B5 LPS (10 nM).

	IC_{50} (μM)		
Comp.	**HEK-Blue**	**HEK293 hMD-2·hTLR4**	**HEK293 mMD-2·mTLR4**
50	3.7 ± 0.4	3.9 ± 1.5	3.3 ± 1.2
70	1.3 ± 0.1	1.4 ± 0.3	0.8 ± 0.2
109	5.0 ± 1.0	0.6 ± 0.02	0.6 ± 0.03
107	0.6 ± 0.05	0.2 ± 0.02	0.2 ± 0.03

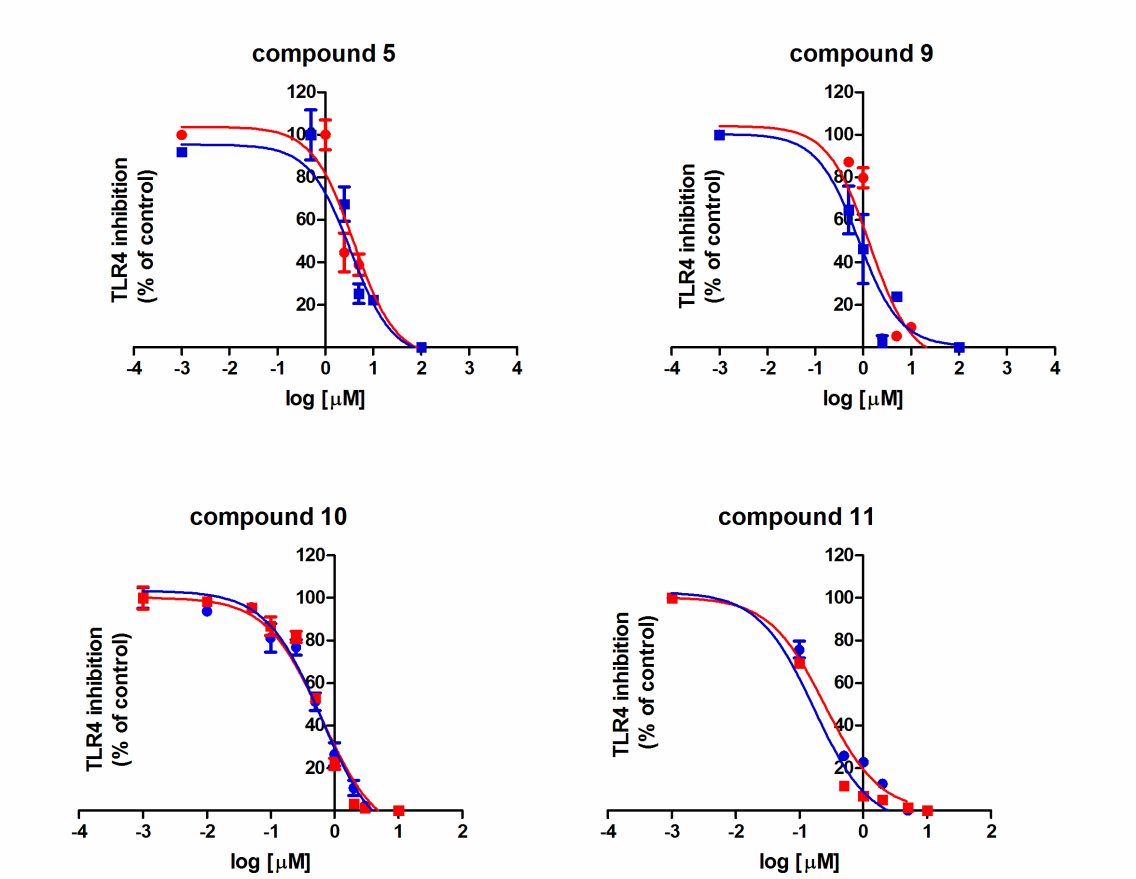


Figure 6.6. Dose-dependent inhibition of LPS-stimulated TLR4 activation by compounds **70**-**107**. HEK293 cells transfected with human MD-2·TLR4 (red) or murine MD-2·TLR4 (blue) were treated with increasing concentrations of compounds and stimulated with LPS (5 nM). Normalized data are representative of three independent experiments.

6.2.3. Evaluation of TLR4 modulation in murine macrophages.

Compounds **70**, **50** and **107** were evaluated on LPS-induced TLR signaling in bone marrow-derived murine macrophages (BMDM), cells naturally expressing TLR4 pathway genes. While compounds **50** and **70** showed very low or no activity in inhibiting TLR4 signaling, compound

107 showed a concentration-dependent inhibition of IL-6 and TNF-α production at concentrations of 1 and 2 μM, while at 0.1 and 0.5 μM they had no effects or the effects were very low (Figure 6.7.).

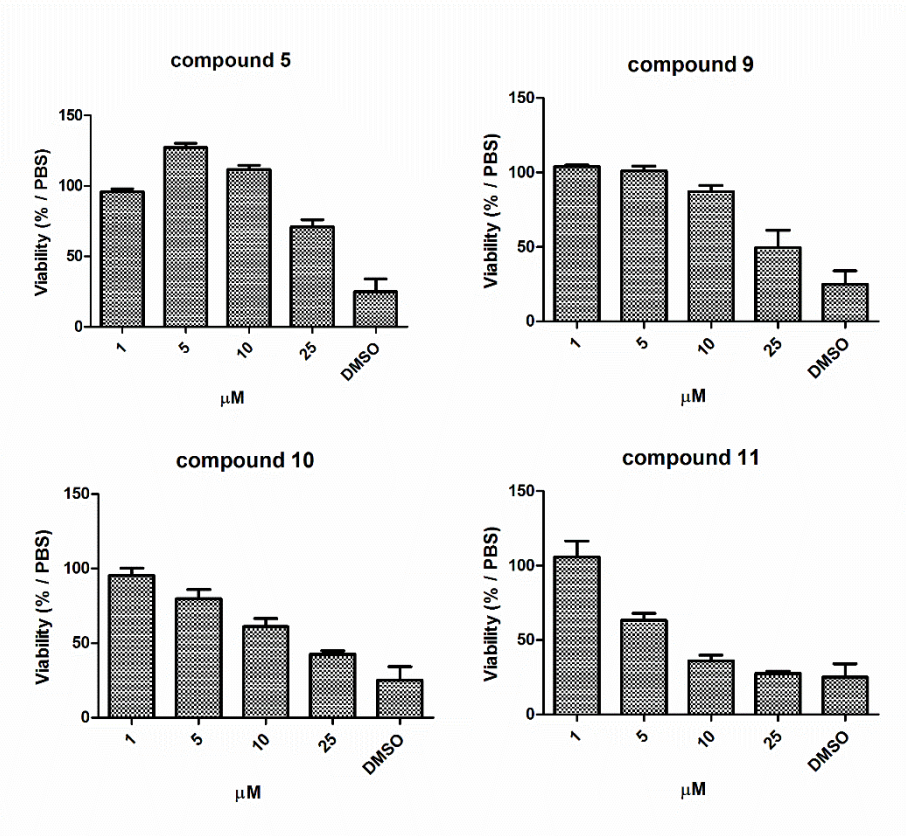


Figure 6.7. HEK-Blue™ cells were treated with increasing concentrations of compounds **50**-**109** and, after overnight incubation, MTT assay was performed. The results were normalized with untreated control (PBS) and expressed as the mean of percentage ± SD of three independent experiments.

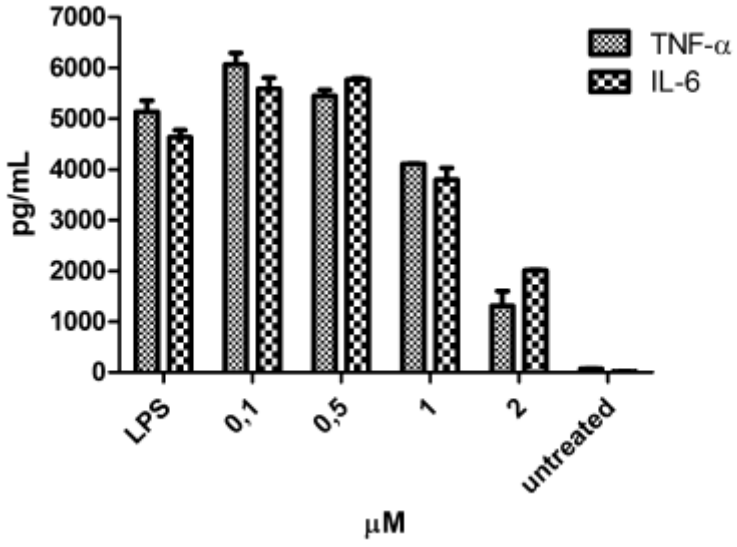


Figure 6.8. BMDM were treated with increasing concentrations (0−2 μM) of compound **107** in RPMI +

FBS 10% in the presence of LPS, administered 1 h after the treatment with **107**. The ELISA assay, performed after overnight incubation, revealed a dose dependent decrease of LPS-induced IL-6 and TNF-α production. Cytokines productions in cells not treated with LPS are reported as negative controls**.**

6.2.4. Preparation and biological activity of gold nanoparticles coated with a selected cationic trehalose amphiphile and evaluation of in vivo activity.

Lipopolysaccharide is an amphiphilic molecule, being present in the form of micellar aggregates when extracted from outer membrane of gram-negative bacteria, in a concentration range very relevant for its biological activity. In a recent work, the group of Prof. Peri demonstrated that the multiple presentation of LPS monomers on metal nanoparticles was a way to potentiate the agonist action, most likely by mimicking de 3D structures of LPS aggregates., the group of Mareque-Rivas obtained similar results by functionalizing stearic acid-coated Quantum Dots with LPS.[34,35] Moreover, the possibility of in vitro or in vivo delivery based on NP is considered advantageous for clinical development, as it can maximize the effectiveness of drugs and minimize the invasiveness and toxic side effects. Surprisingly, no data was found in the literature concerning multivalent presentations of TLR4 *antagonists*. To test the suitability of this approach in the case of cationic glycoamphiphiles, we undertook the preparation and biological evaluation of dodecanethiol-functionalized gold nanoparticles (AuDDT) coated with compound **107**)AuDDT-**107**) by non-covalent interactions. Colloidal gold nanoparticles were prepared by a variation of the Brust-Schiffrin method,[36] resulting in small and low polydispersed nanoparticles, which were further coated by surface adsorption with the trehalose derivative **107**. This coating process preserved the small size with low polydispersity characteristics. Interestingly, after the biological evaluation of AuDDT-**107** in HEK-Blue™ cells, compound **107** retains activity without apparent increase in cytotoxicity. These results can be considered as a proof-of-concept of the possibility of developing nanoparticle systems based on TLR4 antagonist, especially cationic glycolipids, as modulators of TLR4 signaling. On the basis of these results, AuDDT-**107** was further tested on HEK293 cells, exhibiting strong LPS antagonism at very low concentrations of nanoparticle both in the human and murine MD2·TLR4 receptor complex.

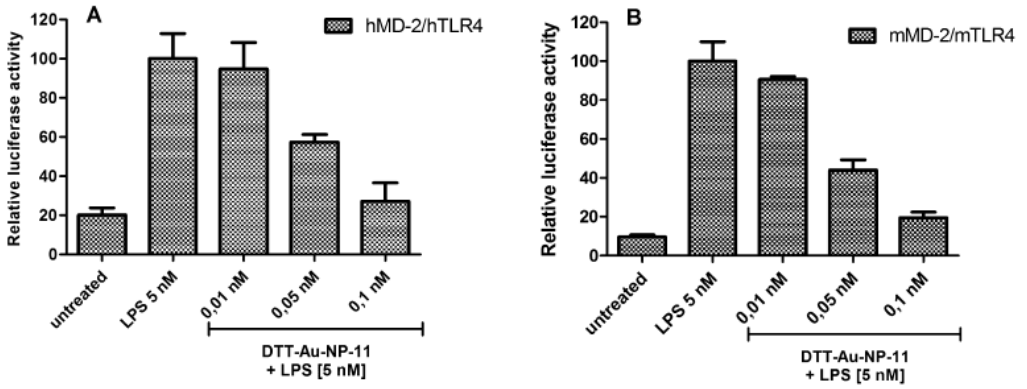


Figure 6.9. Dose-dependent TLR4 antagonism in HEK293 cells treated with AuDDT-**107**. HEK293 cells were transfected with NF-κB-dependent luciferase and constitutive Renilla luciferase reporter plasmids as well as with (A) human or (B) murine MD-2 and TLR4 plasmids. The indicated amount of the AuDDT-**107** was added to the cells, followed 1 h later by stimulation with LPS. Luciferase activity was measured 16 h later.

6.2.5. In vivo activity.

In view of the good results obtained in vitro, the best candidates (**50**, **70**, **109**, **107** and AuDDT-**107**) were selected to be tested in vivo. All candidates (**50** to **107**) potently inhibited LPS-induced immune activation in C57/Bl6 mice. The strongest inhibition profile was exhibited by **107**, which totally inhibited the LPS-induced TLR4 signaling, probing that all single molecule compounds are strong MD-2·TLR4 inhibitors in vitro and in vivo. Unfortunately, AuDDT-**107**, although being still active in cells, showed very high toxicity in mice, preventing their use of the nanoparticles in vivo as TLR4 antagonists carrier .

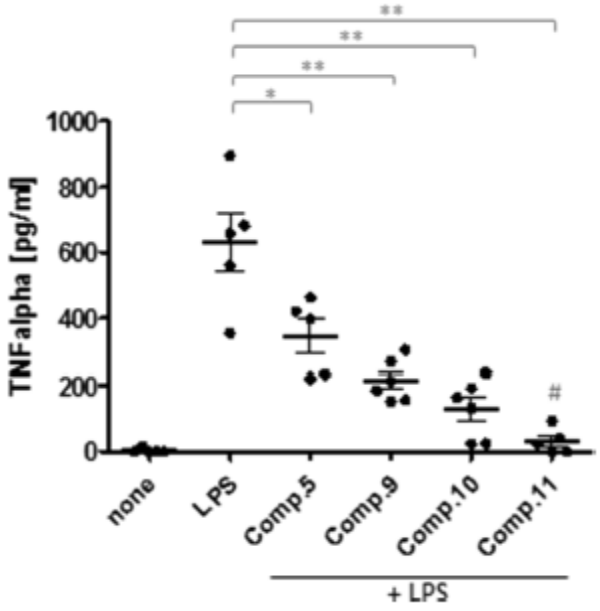


Figure 6.10. In vivo activity of cationic amphiphiles. C57/Bl6 mice were injected intraperitoneally (ip) with the indicated compounds (2×10^{-7} mol/mouse), followed 1 h later by ip injection of LPS (1×10^{-9}

mol/mouse). Three hours later, sera were collected and TNF-α concentration was determined by ELISA (data shown with mean and standard error, N= 5−6) two-tailed t test (* $p < 0.1$; ** $p < 0.01$−compared to ≫LPS≪) (# not significant−compared to ≫none≪).

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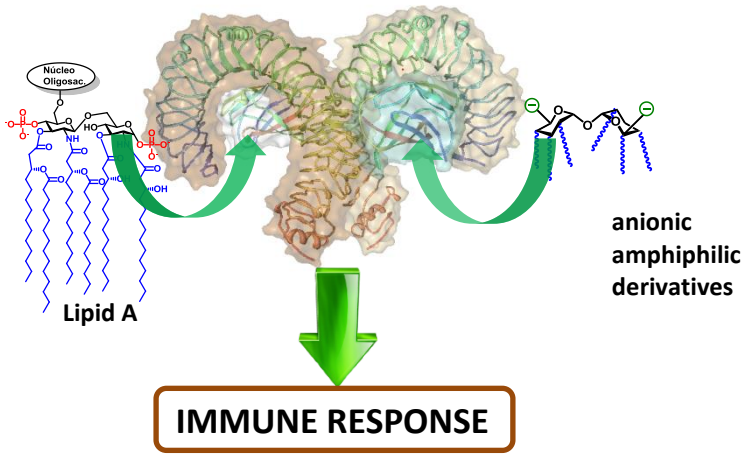
Capítulo 7

Síntesis y caracterización de derivados aniónicos anfifílicos de glucosa y trehalosa.

Abstract: As mentioned in the previous chapter, *E. Coli* Lipid A structure consists in a disaccharidic core with negatively charged phosphate groups and 6 lipophilic chains. Great efforts are being done in the design of Lipid A analogs that mimic the Lipid A structure by interacting with TLR4, acting mainly as agonist, although antagonist activity has also been observed in anionic derivatives. TLR4 agonist can be useful as adjuvants in vaccine development and in cancer immunotherapy. Herein we present a novel family of trehalose-based anionic amphiphilic derivatives carrying carboxylate, sulphate and sulphonate anionic groups in order to determine its profile as TLR4 pathway modulators.



7. Síntesis y caracterización de derivados aniónicos anfifílicos de glucosa y trehalosa.

7.1 Introducción

Como se ha comentado en el capítulo anterior, la modulación (activación/inhibición) de los receptores TLR4 juega un papel muy importante en los mecanismos de respuesta inmune innata.[1] La activación de los receptores TLR4 está asociada a ciertas enfermedades autoinmunes, a desórdenes inflamatorios no infecciosos y a daños neuropáticos, lo que indica un amplio rango de aplicaciones clínicas para el diseño de antagonistas sintéticos de este receptor.[2] Por otro lado, los agonistas (activadores) de TLR4 han mostrado utilidad como agentes adyuvantes en el desarrollo de vacunas[3] y en inmunoterapias contra el cáncer.[4] La mayoría de estos moduladores del TLR4 son moléculas pequeñas relacionadas con el lípido A (Figura 7.1), un lipodisacárido fosforilado cargado negativamente que es el responsable de la activación del TLR4 a través de procesos de reconocimiento específico. En general, los miméticos sintéticos del lípido A son lípidos aniónicos que portan una o dos cargas negativas en grupos fosfatos o grupos isostéricos de los fosfatos (carboxilatos o sulfatos) con un dominio hidrofóbico constituido por cadenas lipídicas (cadenas alquílicas o esteroides) en los que el núcleo disacarídico de β-(1→6)-diglucosamina puede ser sustituido por otro soporte sacarídico o no sacarídico.[5] Estos miméticos aniónicos pueden actuar como antagonistas del TLR4 en proceso proinflamatorios (eritoran,)[6] o como agonistas (ONO1004).[7] Este último compuesto es un derivado de tipo *N*-acil-4-*O*-sulfatoglucosamina que induce de manera eficaz la producción de TNF-α en células tumorales, aunque su baja solubilidad en medio fisiológico conlleva dificultades para su aplicación clínica.

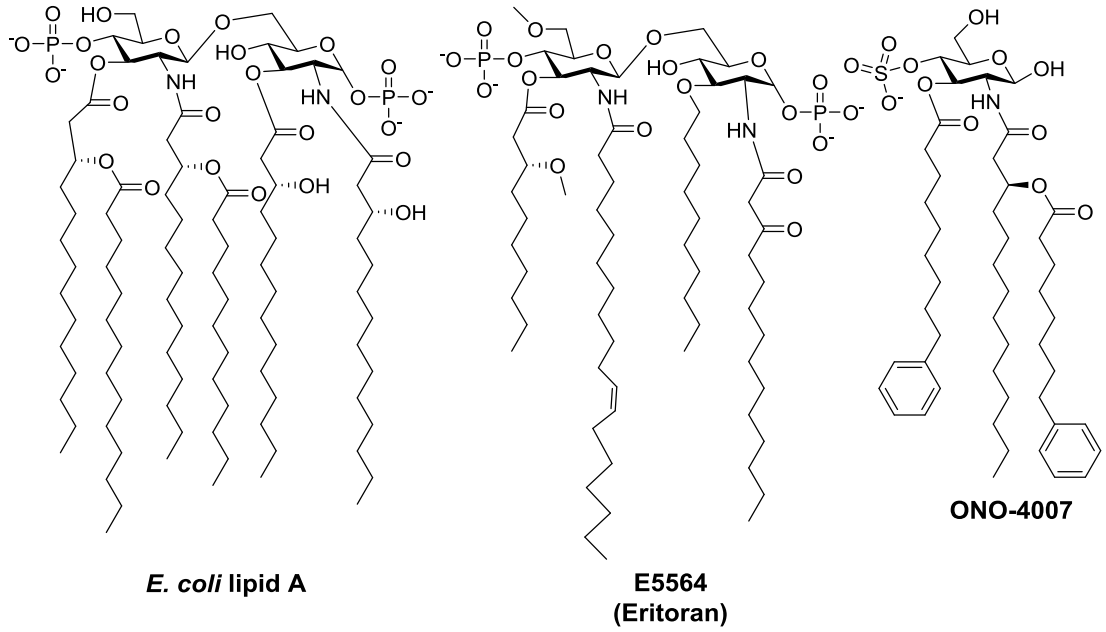


Figura 7.1. Estructuras del lípido A y de antagonistas y agonistas sintéticos.

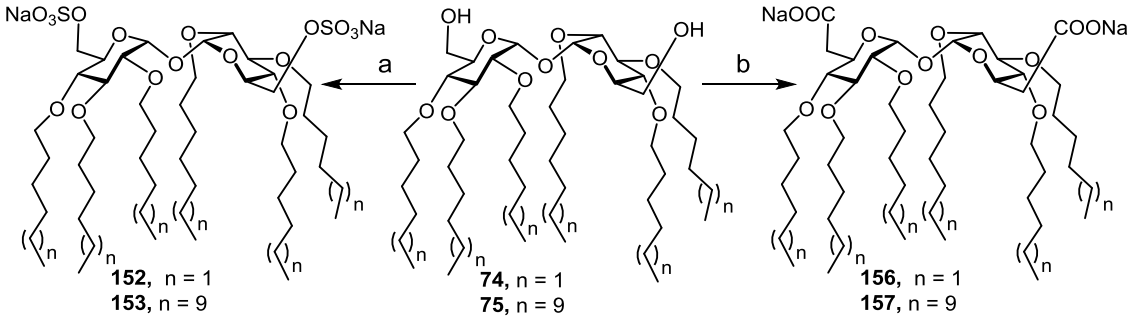
En el capítulo anterior, se han descrito los resultados relativos a la evaluación de una amplia colección de moléculas anfifílicas catiónicas derivadas del metil α-D-glucopiranósido y de la α,α'-trehalosa que ha puesto de manifiesto la capacidad de estos compuestos como moduladores de la actividad del receptor TLR4, tanto en forma de moléculas individuales como tras su presentación multivalente en la superficie de nanopartículas de Au.[8] Resultados anteriores del grupo del Prof. Peri han mostrado que derivados anfifílicos aniónicos sencillos pueden actuar también como moduladores de este receptor.[9] A la vista estos antecedentes, y con objeto de estudiar el efecto de grupos con carga contraria situados sobre estructuras análogas en la inhibición/activación de los procesos mediados por TLR4, se ha abordado en este capítulo la preparación de derivados anfifílicos aniónicos de metil α-D-glucopiranósido y α,α'-trehalosa, estructuralmente relacionados con los derivados catiónicos anfifílicos ya estudiados, que incorporan grupos sulfatos, sulfonatos y carboxilatos en las posiciones primarias de los azúcares y cadenas lipófilas de diferente naturaleza química y longitud en las posiciones secundarias. Estos grupos aniónicos presentan la ventaja de que las reacciones para su incorporación son más eficaces que las reacciones para introducir grupos fosfatos característicos del lípido A. Además, se han examinado las propiedades de autoorganización de los nuevos compuestos sintetizados (CMCs, tamaños hidrodinámicos y carga superficial) se han estudiado. La evaluación de sus propiedades como moduladores de la actividad de los receptores TLR4 está actualmente realizándose en la Universidad de Milano-Bicocca.

7.2. Resultados y Discusión.

7.2.1. Preparación de derivados aniónicos anfifílicas de trehalosa y glucosa.

Para preparación de los derivados de trehalosa aniónicos incorporando cadenas de hexilo y tetradecilo en las posiciones secundarias se partió de los correspondientes precursores hexaalquilados con los hidroxilos primarios libres **74** y **75**. Los derivados de trahalosa 6,6'-di-*O*-sulfato se obtuvieron empleando condiciones de sulfatación directa con el complejo comercial trióxido de azufre-piridina en DMF a 70 ºC (→**152**, **153**, Esquema 7.1.).[10,11] Por otra parte, los derivados de tipo 6,6'-dicarboxilato **156** y **156** se prepararon mediante una secuencia en dos etapas que implica un tratamiento con NaClO/TEMPO ((2,2,6,6-tetrametilpiperidin-1-il)oxidanil) en condiciones de transferencia de fase para obtener el dialdehido intermedio, seguido de oxidación con $NaClO_2$ en presencia de metilbut-2-eno.[12] Los derivados disulfato y dicarboxilato fueron aislados en forma de sales disódicas por tratamiento con resina de

intercambio catiónico Amberlite IRA-120 Na^+ y sus estructuras se confirmaron mediante espectroscopías de IR, RMN, espectrometría de masas y análisis elemental. En la Figura 7.2. se muestran los espectros de ^1H y ^{13}C RMN (300, 75.5 MHz) del dicarboxilato **156**, donde se observa la señal a 174.0 ppm característica del grupo funcional carboxilato.



Esquema 7.1. Síntesis de los derivados aniónicos de trehalosa **152**, **153**, **156** y **157**. Reactivos y condiciones: (a) SO_3·Py, DMF, 40 ºC, 3 h, Ar, 33% y 70%; (b) i. NaBr, TBAB, TEMPO, $NaHCO_3$ sat., CH_2Cl_2, H_2O, NaOCl; ii. ^{t}BuOH, metilbut-2-eno, $NaClO_2$, NaH_2PO_4 79%.

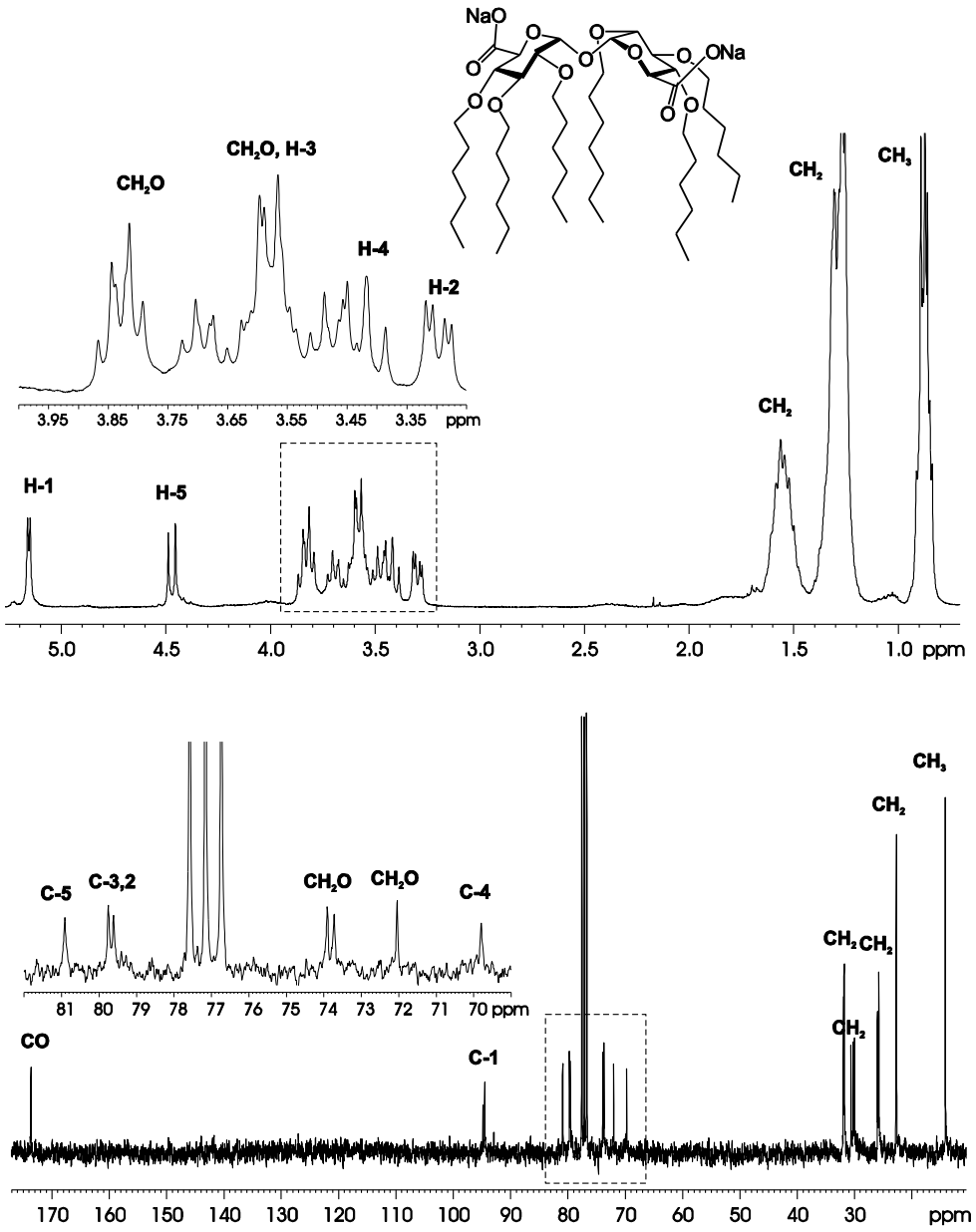
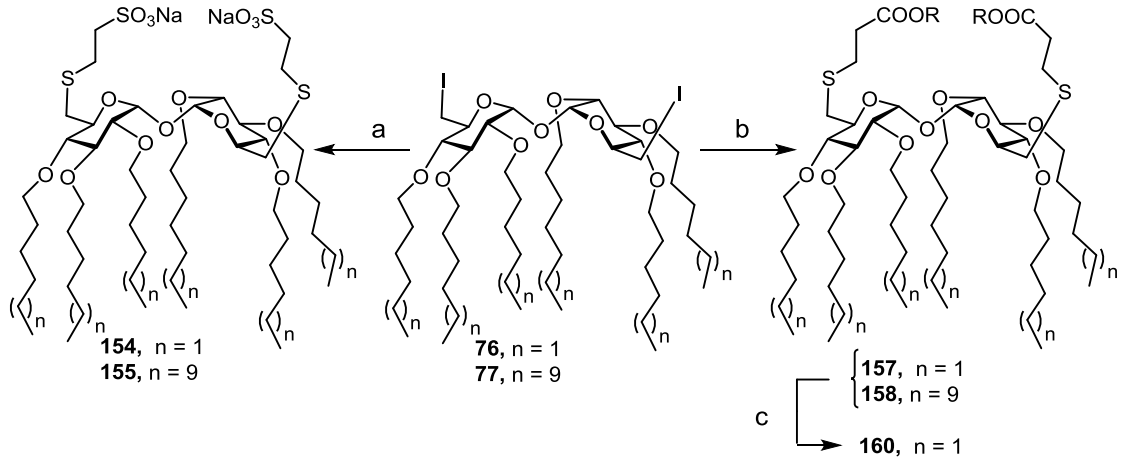


Figura 7.2. Espectros de ^{1}H y ^{13}C RMN (300 MHz, 75.5 MHz, $CDCl_3$) de **156**.

Con objeto de acceder a compuestos en los que los centros aniónicos se encuentren más alejados del soporte sacarídico, se abordó además la preparación de los derivados hexilados y tetradecilados con sustituyentes sulfoniletiltío y carboxietiltío sobre las posiciones primarias. En ambos casos se partió de las correspondientes 6,6'-didesoxy-6,6'-diyodotrehalosas alquiladas **76** y **77** (Esquema 7.2.). La sustitución nucleofílica de los átomos de yodo por 2-mercaptoetanosulfonato sódico en presencia de NaMeO (1 M) proporcionó los derivados aniónicos **154** y **155** con excelentes rendimientos. La reacción análoga con 3-mercaptopropionato de metilo se realizó en presencia de Cs_2CO_3 (→**158**, **159**), puesto que bases más fuertes daban lugar a la saponificació del grupo éster y complicaban la reacción de sustitución. En una segunda etapa se realizó la hidrólisis del diéster **158** en dioxano con NaOH 1

M, aislándose el correspondiente dicarboxilato **160** con excelentes rendimientos (Esquema 7.3.). Sin embargo, la saponificación del éster **159** no permitió obtener el correspondiente dicarboxilato, aunque se observó por ccf la formación del compuesto, debido a procesos de descomposición.



Esquema 7.2. Síntesis de los derivados aniónicos de trehalosa **154**, **155**, **160** y **161**. Reactivos y condiciones: (a) $HS(CH_2)_2SO_3Na$, NaMeO 1 M, DMF, 70 ºC, 16 h, 98% y 62%; (b) $HS(CH_2)_2COOMe$, Cs_2CO_3, DMF, 70 ºC, Ar, 12 h, 76% y 47%; (c) NaOH 1 M, dioxano, 40 ºC, 16 h, 80% y descomposición.

En la Figura 7.3a se muestran los espectros de ^1H y ^{13}C RMN (300, 75.5 MHz) del dipropionato **160**, donde se observa un solo juego de señales de protones para las dos unidades de glucosa de la trehalosa y una sola señal carbono 178.0 ppm del carbonilo del carboxilato, de acuerdo con la simetría C_2 de la molécula. Dado el carácter aniónico de estos derivados, los espectros de masas (ESI-MS) se llevaron a cabo en modo negativo (Figura 7.3b).

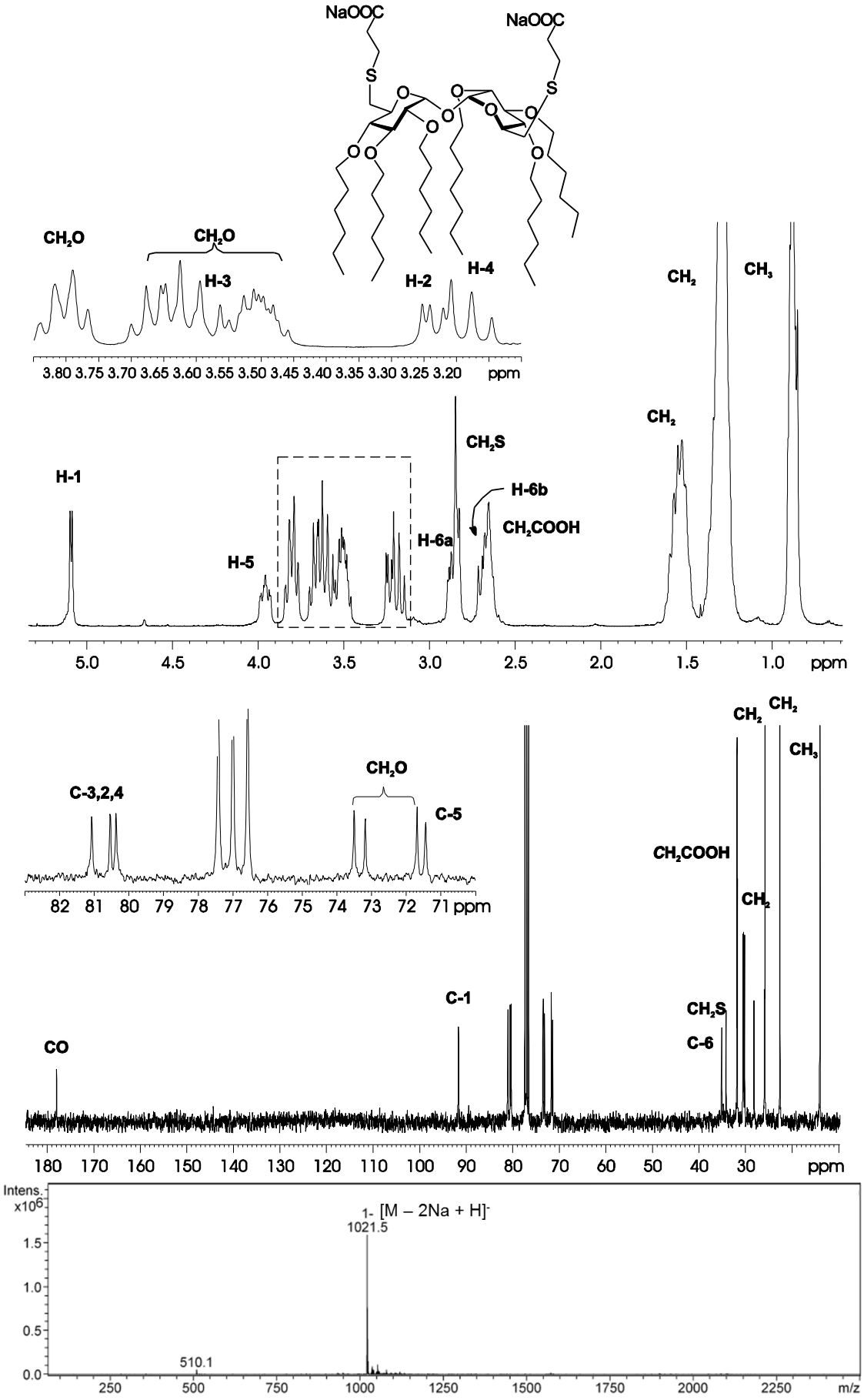
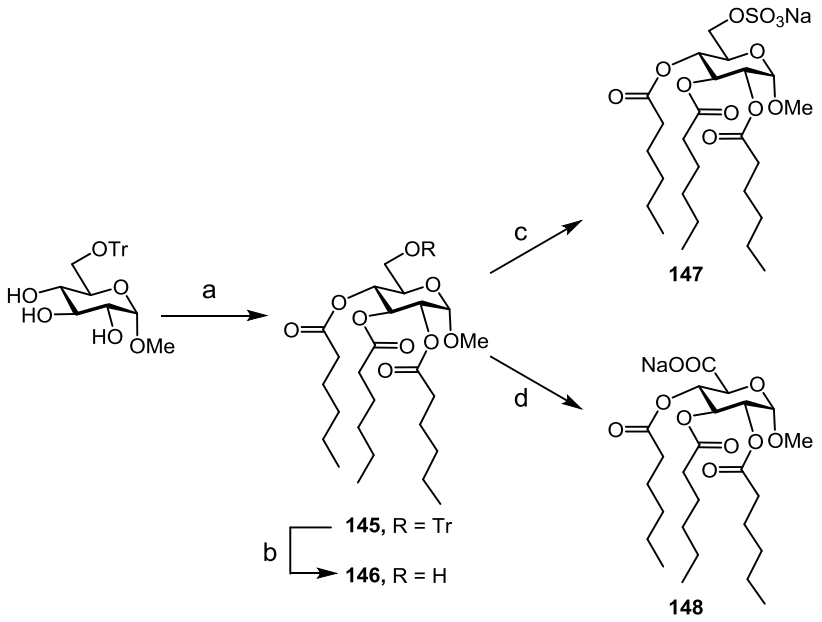


Figura 7.3. Espectros de ^{1}H y ^{13}C RMN (300 y 75.5 MHz, $CDCl_3$) y de EM-ESI de **160**.

Con objeto de disponer de compuestos que permitan evaluar la influencia de naturaleza de las cadenas lipídicas en la actividad de los moduladores del TLR4,[13] se ha abordado además la síntesis de derivados aniónicos anfifílicos en los que la región hidrofóbica estuviera constituida por cadenas de hexanoílo. En primer lugar se preparó el derivado monosacarídico de tipo 6-sulfato **147** a partir del metil 6-*O*-tritil-α-D-glucopiranósido, mediante una ruta en varios pasos que implica la hexanoilación de los hidroxilos secundarios (→**145**), la hidrólisis ácida de los grupos tritilo (→**146**) y la sulfatación directa de los hidroxilos primarios con el complejo comercial trióxido de azufre-piridina (→**147**). Esta última reacción se llevó a cabo de modo muy eficaz por activación con radiación de microondas,[14] ya que la sulfatación de **146** en DMF a 70 ºC condujo a rendimientos bajos del derivado **147** (Esquema 7.3). Para la obtención del derivado carboxilato **148** y con objeto de evitar el medio básico requerido en el tratamiento con TEMPO, que es incompatible con los grupos ésteres, se utilizó un método de oxidación diferente al descrito para **154** y **155**. El tratamiento del diol **146** con reactivo de Dess-Martin, seguido de la oxidación *in situ* del dialdehido intermedio con clorito de sodio en *terc*-butanol condujo a **148** con buen rendimientos (73%, Esquema 7.3).[10]



Esquema 7.3. Síntesis de los derivados aniónicos de metil glucósido **147** y **148. Reactivos y condiciones: (**a) Anhídrido hexanoico, DMAP, DMF, Ar, rt, 4 h, 88%; (b) PTSA·H_2O, 1:1 DCM-MeOH, 40 ºC, 2 h, 80%; (c) SO_3·Py, Py, Ar, 60 ºC, microondas 20 w, 15 min, 100%; (d) i. Dess-Martin Periodinano (DMP), DCM, NaOCl; ii. ^{t}BuOH, metilbut-2-eno, $NaClO_2$, NaH_2PO_4, 73%.

Las estructuras de los derivados hexanoilados aniónicos de metil glucósido y sus precursores **145**, **146**, **147** y **148** se confirmaron mediante espectroscopía de IR, RMN, espectrometría de masas y análisis elemental. En la Figura 7.4 se muestran los espectros de ^{1}H y ^{13}C RMN (300,

75.5 MHz) del sulfato **147**, donde se observa el desapantallamiento de las señales de los protones H-6a y H6b a 4.15 y 4.00 ppm, respectivamente, y de la señal de C-6 a 66.1 ppm.

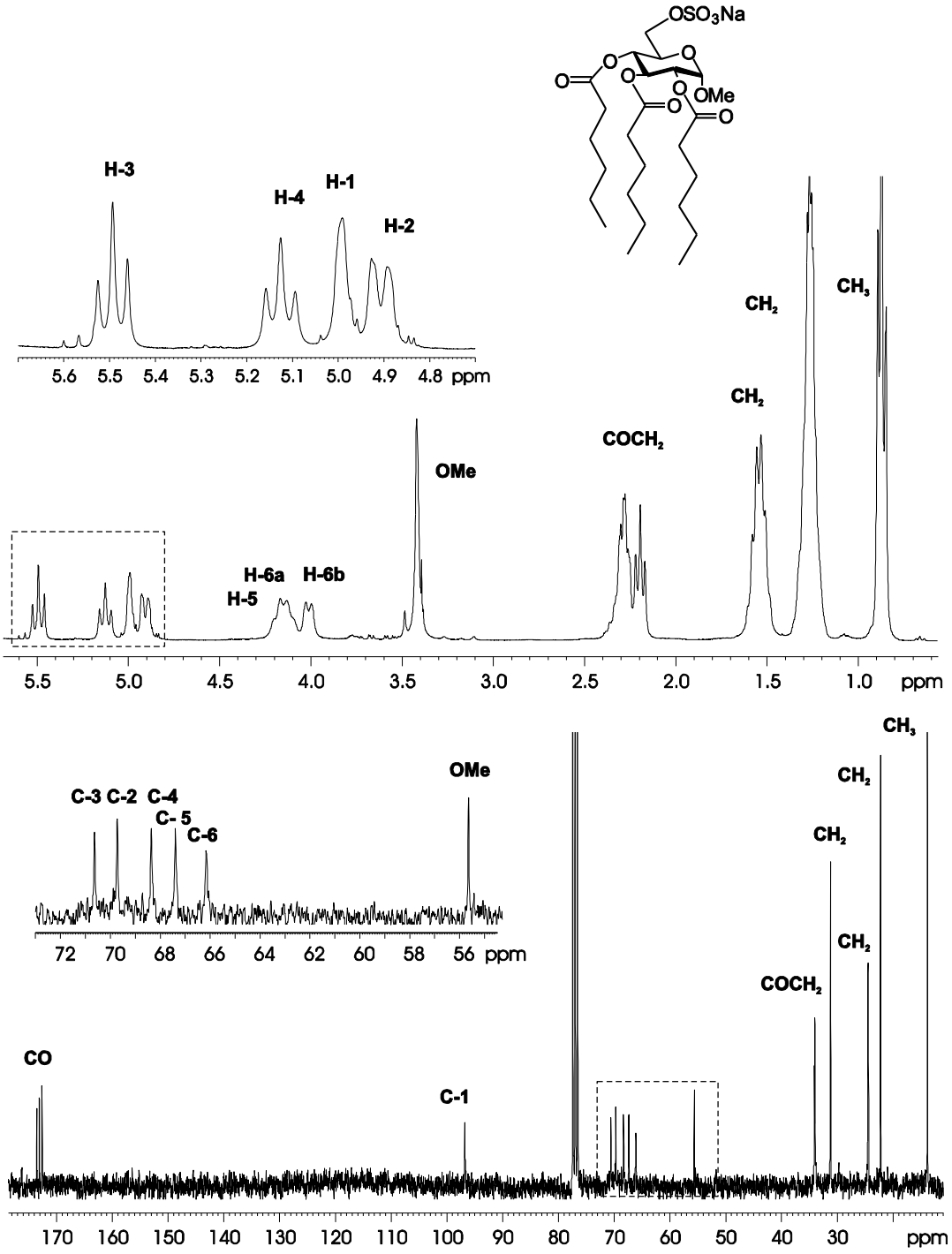
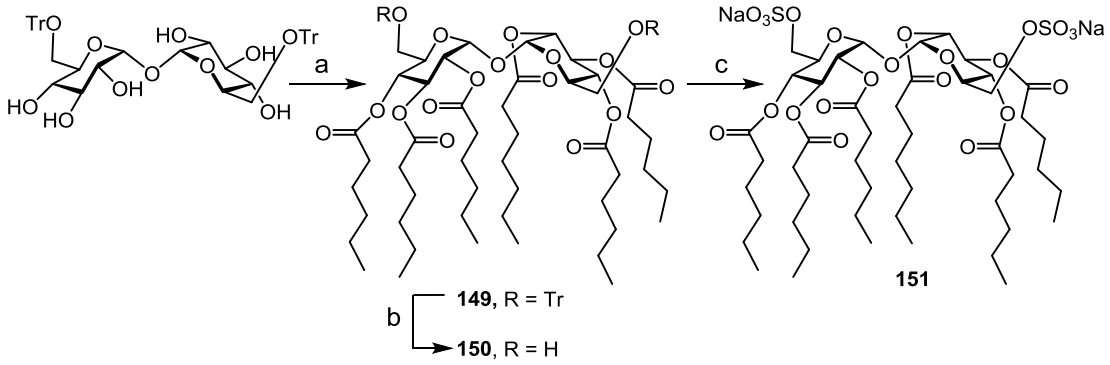


Figura 7.4. Espectros de ^{1}H y ^{13}C RMN (300 y 75.5 MHz, $CDCl_3$) y EM-ESI de **147**.

Se ha preparado también el derivado de trehalosa hexanoilado 6,6'-disulfato **151** siguiendo una secuencia de reacciones análoga a la utilizada para la preparación de **147**, esto es, hexanoilación de los hidroxilos secundarios (→**149**), hidrólisis ácida de los grupos tritilo (→**150**) y sulfatación directa de los hidroxilos primarios con el complejo comercial trióxido de azufre-piridina mediante activación con microondas[14] (→**151**, Esquema 7.4). Como cabía

esperar, el espectro de ^{1}H RMN de **151** muestra un único juego de señales para la glucosa, mientras que el espectro de ^{13}C RMN de muestra señales de los carbonilos de éster a 174.2-173.8 ppm y un desplazamiento a campo alto de la señal de C-6 (55.1 ppm) que indica la presencia del anión sulfato en esa posición (Figura 7.5).



Esquema 7.4. Síntesis del derivado aniónico de trehalosa **151**. Reactivos y condiciones: (a) Anhídrido hexanoico, DMAP, DMF, Ar, rt, 4 h, 62%; (b) PTSA·H_2O, 1:1 DCM-MeOH, 40 ºC, 2 h, 66%; (c) SO_3·Py, Py, Ar, 60 ºC, microondas 20 w, 15 min, 100%.

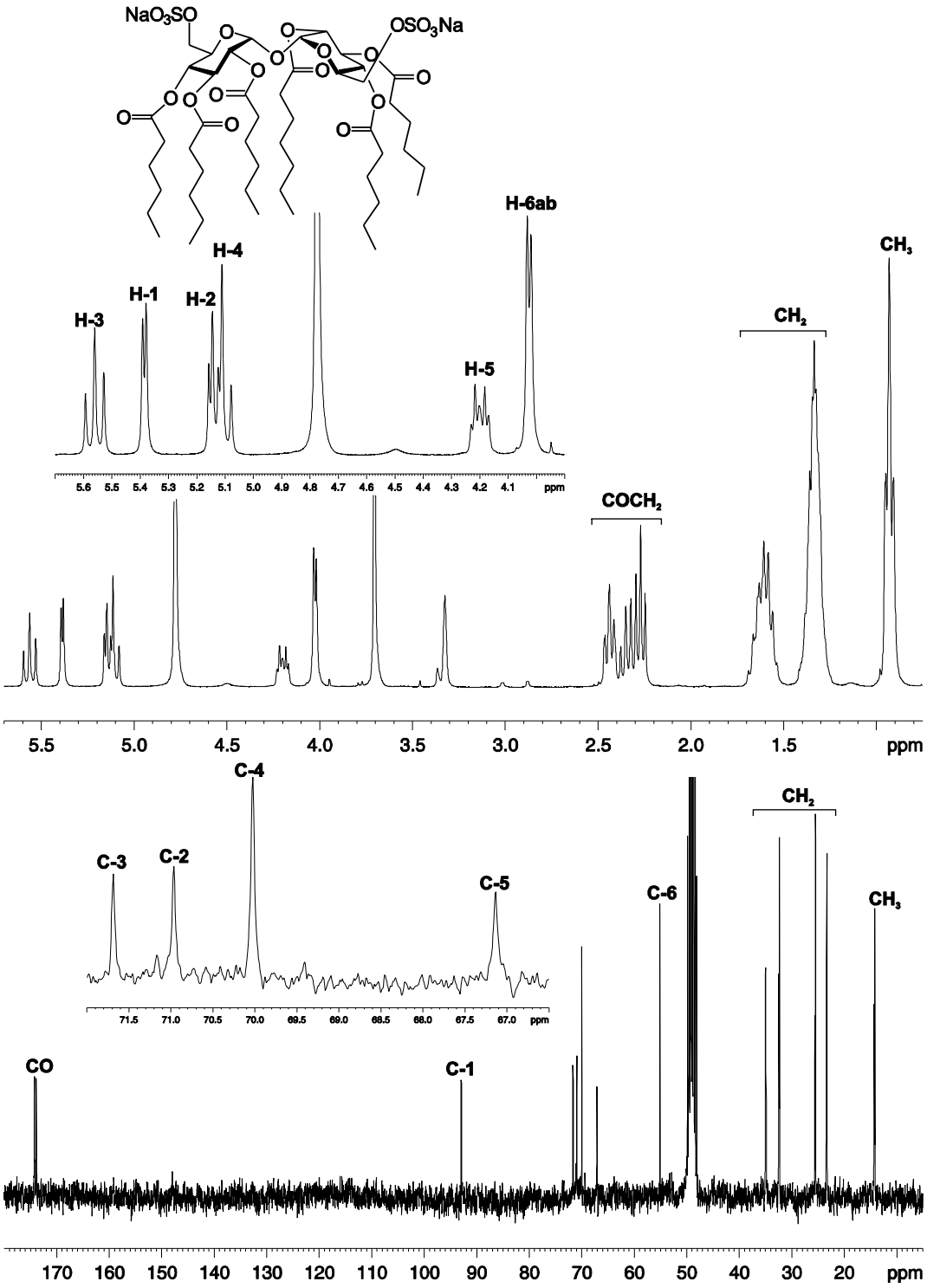


Figura 7.5. Espectros de ^{1}H y ^{13}C RMN (300 y 75.5 MHz, CD_3OD) de **151**.

7.2.2. *Evaluación de las propiedades de autoorganización de los derivados aniónicos anfifílicos de trehalosa y glucosa.*

Para la evaluar la anfifilicidad de nuestros compuestos y su capacidad de autoorganizarse en medio acuoso se han realizado valoraciones fluorimétricas utilizando pireno como sonda fluorescente,[15] con objeto de determinar la concentración micelar crítica, y se ha medido el

tamaño y el potencial zeta de los agregados utilizando a concentraciones diferentes (5 y 50 μM; Figura 7.6). Para el estudio de las propiedades de autoorganización se han preparado nanopartículas utilizando el método de hidratación de película fina descrito en los métodos generales. Los valores de CMC, tamaño hidrodinámico y potencial zeta se recogen en la Tabla 7.1.

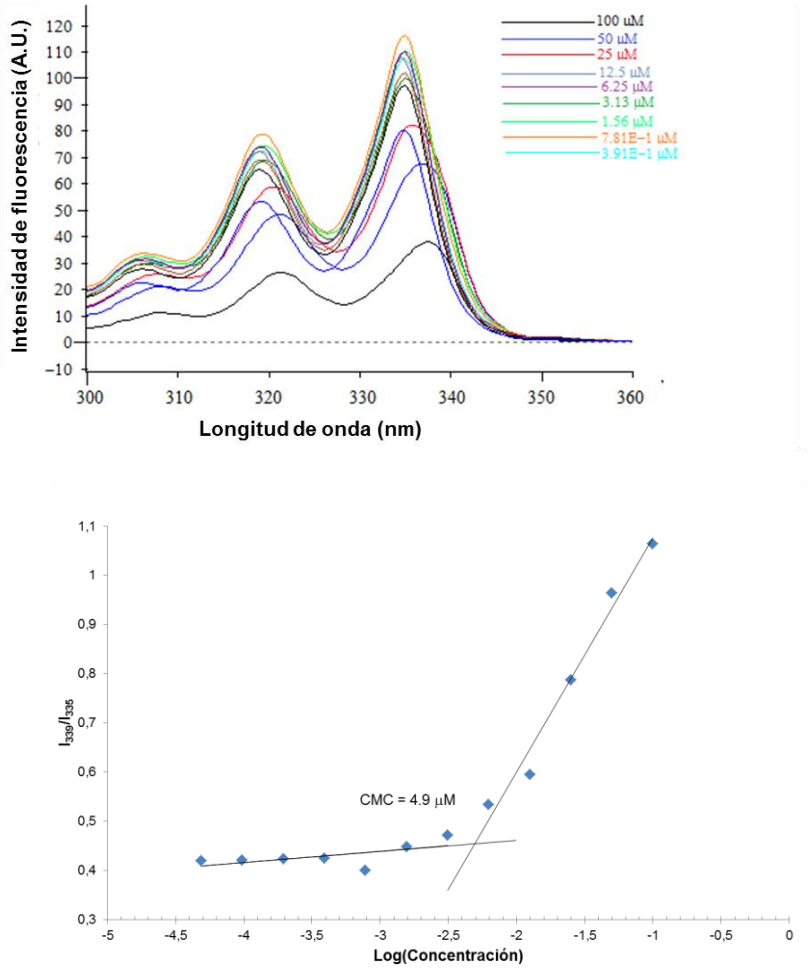


Figura 7.6. Determinación de la concentración micelar crítica del compuesto **153**. (a) Espectros de excitación de la fluorescencia de pireno (λem 375 nm) en agua en presencia de **151**. (b) Determinación de CMC de **153**.

En general, los derivados anfifílicos aniónicos alquilados **JRL228**, **152**, **153**, **154** y **160**, **6** muestran valores de CMCs menores (7.8-4.6 μM) que los derivados hexanoilados **151**, **147** y **148** (Tabla 7.1). Sólo el derivado alquílico **156** mostró un valor de CMC un orden de magnitud superior (39.0 ± 2.8 μM). En el caso de los derivados hexanoilados, los derivados de metil glucósido **147** y **148** muestran valores superiores de CMCs que el derivado de trehalosa **151**.

Tabla 7.1. Concentración micelar crítica (μM), tamaños hidrodinámicos (nm), potenciales ζ (mV) e índices de polidispersidad (PI) de los derivados aniónicos anfifílicos.

Comp.	CMC (mM)	Tamaño conc. 5 μM	Potencial ζ conc. 5 μM	PI	Tamaño conc. μM 50	Potencial ζ conc. 50 μM	PI
155	5.7±0.4	n.d.*	n.d.	n.d.	n.d.	n.d.	n.d.
153	4.8±0.2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
152	7.8±1.0	n.d.	n.d.	n.d.	399.7±28.2	-31.4±3.8	0.254
154	4.8±1.0	n.d.	n.d.	n.d.	420.1±54.2	-42.1±0.0	0.326
160	5.5±0.4	n.d.	n.d.	n.d.	281.4±15.2	-32.1±0.7	0.333
156	39.0±2.8	452.5±50.8	-57.3±5.7	0.515	226.6±6.8	-56.2±0.9	0.286
157	4.6±1.4	340.1±20.9	-50.8±2.0	0.396	269.9±5.5	-61.5±0.5	0.514
151	45.5±9.2	408.3±79.5	-61.5±0.5	0.514	522.3±66.3	-45.7±3.14	0.499
147	204±35.4	326.9±33.7	-52.5±5.9	0.330	360.4±20.52	-36.5±2.3	0.396
148	251±23.0	308.7±30.9	-58.6±1.5	0.367	269.1±19.9	-59.6±3.02	0.344

*n.d.: No se detecta formación de nanopartículas.

Los resultados obtenidos, recogidos en la tabla 7.1 indican que los derivados hexanoilados tienen una mayor tendencia a autoorganizarse en agua que los derivados alquilados. En todos los derivados aniónicos en los que se formaron nanopartículas los diámetros hidrodinámicos estaban entre 226-452 nm con polidispersidades de moderadas a bajas (Figura 7.7) y con cargas superficiales negativas.

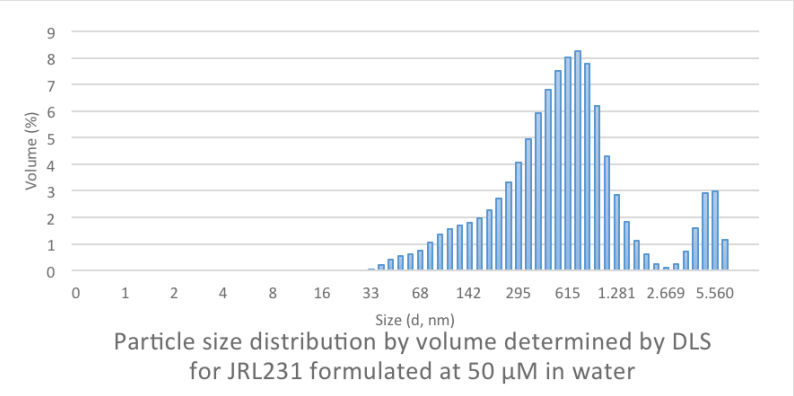


Figura 7.7. Distribución de tamaños de partícula en %volumen determinado por DLS para el compuesto **152** formulado a 50 µM en H_2O.

La evaluación de la capacidad de estos compuesot snaiónicos anfifílicos de modular la actividad con el TLR4 está realizándose en la Universidad de Milán-Bicocca en el grupo de Profesor Peri. Los datos nos deben permitir establecer si compuestos structuralmente relacionados pero con cargas opuestas actúan de manera deiferente (e.g. agonistas/antagonistas) y si esta actividad se modifica en función de que actúen como moléculas individuales o asociadas en sistemas nanoparticulares.

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Capítulo 8
Conclusiones

8. Conclusions.

The ensemble of results obtained in this Thesis leads to the following conclusions:

FIRST. The results included in this Thesis provides a proof of concept of the suitability of the βCD—mannosyl dendron conjugate $(ManS)_3$-βCD as a macrophage-targeted delivery system for sp^2-iminosugar-type amphiphilic pharmacological chaperones against Gaucher disease. The monosubstituted but multivalent conjugate structure of the carrier prototype has been purposely conceived to keep the inclusion capabilities towards the hydrophobic moieties of the PC candidates **6S-NOI-NJ** and **6S-NAdB-NJ**, bearing octyl and adamantyl moieties, respectively, while warranting efficient mannose-specific lectin recognition abilities. Both hypotheses have been first demonstrated for commercial β-glucosidases and concanavalin A (Con A) lectin and further validated in human β-glucocerebrosidase and human macrophage mannose receptor (MMR, recombinant). Complexation of the pharmacological chaperones by $(ManS)_3$-βCD preserves their chaperoning potential. The corresponding PC complexes specifically recognize the MMR at the surface of macrophages, the cell type that is mostly affected in GD patients, and this recognition phenomenon elicits macrophage internalization. The combination of macrophage targeting abilities and efficient PC transfer to GCase may provide a means for improving the therapeutic outcome of pharmacological chaperone treatments for Gaucher disease and warrants further in vivo evaluation.

SECOND. Taking advantage of two of the most efficient "click" methodologies, namely the Cu (I)-catalyzed azide-alkyne coupling reaction and the thiourea-forming reaction, in this Thesis we have prepared a small library of amphiphilic polycationic derivatives using methyl α-D-glucopyranoside and trehalose as scaffolds. These flexible

synthetic strategies allow the sequential installation of cationic clusters on the primary hydroxyl groups of the carbohydrates and hydrophobic (acyl, alkyl) substituents on the secondary hydroxyl groups (skirt-shaped derivatives) or the reverse" architecture, with the hydrophobic groups at the primary positions and the polycationic groups at the secondary face (jellyfish-shaped derivatives). The influence of the hydrophilic/hydrophobic balance and the investigation of their self-assembling capabilities in aqueous environment have been thoroughly studied by different techniques.

THIRD. New cationic amphiphiles based on monosaccharide and disaccharide glycolipid scaffolds have been designed, synthesized and their capacity to modulate TLR4 activation and signaling evaluated. A glucose-based compound and several trehalose-based compounds were found to be active in inhibiting the LPS-triggered TLR4 activation and signaling in HEK cells transfected with human and murine MD-2·TLR4 complexes, with IC_{50} values ranging from about 5 to 0.2 μM with a low cell toxicity. The inhibition potency was very similar for the human and murine systems, similarly to that encountered for the very efficient TLR4 antagonist Eritoran® and differently from the natural TLR4 antagonist lipid IVa. Additionally, these compounds significantly inhibited LPS-triggered IL-6 production in mice. Since these compounds are active *in vitro* and *in vivo*, and show low toxicity, they represent good leads for the development of drugs targeting TLR4 signaling.

The ensemble of data suggests some general structure-activity relationships in this type of compounds: i) the presence of acyl lipophilic chains at the hydrophobic domain seems to be a primary requisite since all compounds with ether bonds are inactive; and ii) the trehalose scaffold favors the biological activity, probably by providing a well-ordered facial amphiphilic character. Absorption of the most effective trehalose derivative on gold nanoparticles preserves the TLR4 antagonist activity in cells, but the resulting systems exibit a high toxicity that could prevent their use *in vivo*. Finally, the experimentally

determined CMC values for the cationic glycolipids suggest that the active species are the non-aggregated monomers. This very likely means that specific molecular interaction with CD14 and MD-2 receptors regulate the TLR4 activity of these compounds.

FOURTH. A comparative study on the DNA complexation and cell transfection capabilities of cationic amphiphiles built on D-glucose, α,α'-trehalose and α,α'-trehalose-derived cyclooligosaccharide (cyclotrehalans, CTs) scaffolds revealed that the later are much more efficient as gene vectors. CTs featuring separated multihead and multitail cationic and lipophilic domains can be accessed by a convergent synthesis in which the final step is a high yielding macrocyclisation reaction. Our data support the superiority of multifunctional macrocyclic over open structures as platforms to build facial amphiphiles capable of forming nanocomplexes with DNA after self-assembling. Polycationic amphiphilic CTs (paCTs) represent thus an alternative to polycationic amphiphilic cyclodextrins (paCDs) as molecularly well-defined compounds for gene delivery applications.

FIFTH. Amphiphilic anionic derivatives of methyl α-D-glucopyranoside and trehgalose analogs of lipid A have been efficiently prepared using microwave-assisted sulphatation reactions, nucleophilic displacements with sulphonates or oxidation reactions of primary hydroxymethyll groups to carboxylates. Structure-activity preliminary studies show that some of these derivatives are able to interact with TLR4 behaving as antagonists of lipid A.

8. Conclusiones.

El conjunto de resultados obtenidos en esta Tesis permite extraer las siguientes conclusiones:

PRIMERA. Se ha comprobado la idoneidad del derivado manosilado $(ManS)_3$-βCD como un sistema de transporte eficaz a macrófagos de chaperonas farmacológicas para la enfermedad de Gaucher con estructura de sp^2-iminoazúcares anfífilicas. La estructura del transportador monosustituido y multivalente ha sido diseñada para favorecer las propiedades de inclusión de los sustituyentes hidrofóbicos de las chaperonas candidatas **6S-NOI-NJ** y **6S-NAdB-NJ** al mismo tiempo que se garantiza la capacidad de reconocimiento por lectinas específicas de manosa. Los dos objetivos se han demostrado usando β-glucosidasas y lectina Con A comerciales y se han validado con β-glucocerebrosidasa humana y lectina MMR humana (recombinante). La complejación de las chaperonas con $(ManS)_3$-βCD preserva su actividad, los correspondientes complejos reconocen específicamente a los receptores MMR de la superficie de los macrófagos, el tipo de célula más afectado en pacientes de GD, y este fenómeno permite la internalización en el macrófago.

SEGUNDA. Se ha preparado una colección de deerivados policatiónicos anfifílicos derivados del α-D-glucopiranósido de metilo y de trehalosa haciendo uso de dos metodologías muy eficaces de tipo "click": la reacción de acoplamiento azida-alquino catalizada por Cu(I) y la reacción de formación de tioureas. La flexibilidad de estas estrategias sintéticas permite la incorporación secuencial de clústeres catiónicos en los hidroxilos primarios de los carbohidratos y sustituyentes hidrofóbicos (acilo, alquilo) en los hidroxilos secundarios (derivados tipo "falda") o una distribución opuesta con los hgrupos hidrofóbicos en las posiciones primarias y los grupo policatiónicos en las posiciones secundarias (derivados tipo "medusa"). Este conjunto de derivados anfifílicos

nos ha permitido estudiar la influencia del balance hidrófilo/hidrófobo en las propiedades de auto-ensamblado utilizando diversas técnicas.

TERCERA. Se han diseñado y preparado moléculas catiónicas anfifílicos basadas en monosacáridos y disacáridos con objeto de modular la activación del receptor TLR4. Un derivado de glucosa y varios derivados de trehalosa han mostrado actividad como inhibidores de la activación del TLR-4 promovida por LPS en células HEK con valores de IC_{50} en el rango micromolar bajo y una toxicidad muy baja. Además, los derivados preparados inhiben la producción de IL-6 promovida por LPS en ratones. Los valores de CMC de los glicolípidos catiónicos preparados sugieren que son activos como especies monoméricas.

CUARTA. Se ha abordado una aproximación novedosa a la preparación de plataformas macrocíclicas monomoleculares capaces de condensar y complejar ácidos nucleicos en sistemas nanoparticulares con capacidad de transfección. A partir del disacárido natural α,α'-trehalosa y empleando una síntesis convergente se han preparado macrociclos (ciclotrehalanas, CTs) con dominios lipofílicos y catiónicos separados que se comportan como sistemas de transfección eficaces frente a diversas líneas celulares.

QUINTA. La preparación de derivados aniónicos anfifílicos de glucosa y trehalosa análogos del lípido A se ha llevado a cabo empleando reacciones de sulfatación catalizada por microondas, desplazamientos nucleofílico con sulfonatos o por oxidación. Estudios preliminares de estructura-actividad indican que algunos de estos compuestos interaccionan xon el TLR4 actuando como antagonistas del lípido A.

Chapter 9

Experimental part

9. Experimental Part.

9.1. General Methods.

Thin-layer chromatography was carried out on aluminum sheets coated with *Sílica Gel 60 F_{254} Merck* (0.25 mm), with visualization by iodine vapour, UV light (λ 254 nm) and by charring with 10% H_2SO_4 in ethanol; ninhydrin 0.1% in ethanol; Mostain (20 g of ammonium (VI) molibdate·4 H_2O; 0.4 g of $Ce(SO_4)_2 \cdot H_2O$ and 10% H_2SO_4 in 400 mL of H_2O); 5% phosphomolybdic acid in EtOH and heating at 100 ºC.

Column chromatography was performed on Chromagel (SDS silice 60 AC.C 35-70 μm or 70-200 μm).

Optical rotations were measured at room temperature in 1 cm or 1 dm tubes on a Jasco P-2000 polarimeter using Na line (λ 589 nm), 0.5-1% (w/v) solutions.

Elemental analyses were performed at the Instituto de Investigaciones Químicas (Sevilla, Spain) using an elemental analyser *Leco CHNS-932* o *Leco TruSpec CHN*.

IR spectra were recorded on a Jasco FT/IR-4100 (ATR) spectrometer.

UV spectra were recorded on a Jasco UV-630 spectrometer at 25 ºC.

^{1}H (and ^{13}C NMR) spectra were recorded at 300 (75.5 for ^{13}C), 400 (100.6 for ^{13}C) and 500 (125.7 for ^{13}C) MHz with, respectively, *Bruker AVANCE 300, Bruker AMX400* and *Bruker AVANCE DRX 500* spectrometers. 2D COSY (*Correlated Spectroscopy*) and ^{1}H-^{13}C (HMQC, *Heteronuclear Multiple-Quantum Coherence experiment*) experiments were used to assist on NMR assignments. $CDCl_3$, CD_3OD, CD_3CN, acetone-d_6 and D_2O

have been used as solvents.

For **electrospray** mass spectra, 0.1 pM sample concentrations were used, the mobile phase consisting of 50% aq acetonitrile at 0.1 mL min^{-1} and were obtained with a Bruker Esquirre 6000.

NMR titration experiments. Association constants (*K*as) were determined in D2O at 313 K by measuring the proton chemical shift variations in the 1H NMR spectra of a solution of the βCD derivative in the presence of increasing amounts of the corresponding chaperone.

In a typical titration experiment, a solution of the host (ManS)3-βCD (1.63 mM) in D2O was prepared, a 500 μL aliquot was transferred to a 5-mm NMR tube, and the initial NMR spectrum was recorded. A concentrated solution (ca. 25 mM) of the chaperone 6S-NOI-NJ and 6S-NAdB-NJ in the previous solution was prepared in order to maintain the host concentration constant all throughout the titration experiment. 10 μL aliquots of this solution were sequentially added to the βCD solution and the corresponding NMR spectra recorded until 90-100% complexation of the guest had been achieved. The chemical shifts of the diagnostic signals obtained at 10-15 different host-guest concentration ratios were used in an iterative least-squares fitting procedure

Isothermal titration calorimetry (ITC) ITC experiments were performed in a multichannel thermal activity monitor (TAM) isothermal heat conduction microcalorimeter (Thermometric AB 2277/201, Järfälla, Sweden) equipped with a 1.1 mL titration vessel. The calorimeter was thermostated at 25 ± 0.5 °C. The vessel was loaded with 0.8 mL of protein solution using a Hamilton syringe, thermostated at 25 °C and continuously stirred at 60 rpm. The CD derivative 3 was injected by a computer controlled syringe pump (Hamilton Microlab M). Injections were made over a period of 10 s with intervals of 6 min. The experiment was monitored and analyzed using Digitam

4.1 software (Thermometric). To minimize dilution artifacts, the ligand was dissolved in the same dialysis buffer as the protein. The errors are provided by the software from the best fit of the experimental data to the model of equal and independent sites, and correspond to the standard deviation in the fitting of the curves. A separate experiment was performed to determine the heat of dilution of the ligand in the dialysis buffer. Reported data are the mean of two measurements made with different protein preparations; experiments were repeated more than twice if the obtained parameters were not congruent.

General procedure for the inhibition assay against the commercial enzymes. Inhibition constant (Ki) values were determined by spectrophotometrically measuring the residual hydrolytic activities of the β-glucosidases (from bovine liver or almonds; Sigma) against p-nitrophenyl β-D-glucopyranoside. Each assay was performed in phosphate buffer at the optimal pH for the enzymes. The reactions were initiated by addition of enzyme to a solution of the substrate in the absence or presence of various concentrations of iminosugar or iminosugar:CD complex (prepared by freeze-drying equimolecular mixtures of each partner). The mixture was incubated for 10–30 min at 37 °C and the reaction was quenched by addition of 1 M Na2CO3. Reaction times were appropriate to obtain 10–20% conversion of the substrate in order to achieve linear rates. The absorbance of the resulting mixture was determined at 405 nm. Approximate values of Ki were determined using a fixed concentration of a substrate (around the KM value for the different β-glucosidases) and various concentrations of an inhibitor. Full Ki determinations and enzyme inhibition mode were determined from the slope of Lineweaver–Burk plots and double reciprocal analysis.

Lysosomal enzyme activity assay. Lysosomal enzyme activities in cell lysates were determined as described previously.40 Briefly, cells were scraped in ice-cold 0.1% Triton X-100 in water. After centrifugation (6000 rpm for 15 min at 4 °C) to remove insoluble

materials, protein concentrations were determined using a protein assay rapid kit (Wako, Tokyo, Japan). The lysates were incubated at 37 °C with the corresponding 4-methylumbelliferyl β-D-glycopyranoside solution in 0.1 M citrate buffer (pH 4). The liberated 4-methylumbelliferone was measured with a fluorescence plate reader (excitation 340 nm; emission 460 nm; Infinite F500, TECAN Japan, Kawasaki, Japan). For enzyme inhibition assay, cell lysates from normal skin fibroblasts were mixed with the 4-methylumbelliferyl β-D-glycopyranoside substrates in the absence or presence of increasing concentrations of the pharmacological chaperones 6S-NOI-NJ and 6S-NAdB-NJ or their corresponding 1 : 1 complexes with the mannosylated carrier (ManS)3-βCD.

Cell culture and GCase activity enhancement assay. Human skin fibroblasts were cultured in Dulbecco's modified Eagle's medium–10% foetal bovine serum at 37 °C under a humidified atmosphere containing 5% CO2. One control cell line (H37) and three lines of GD cells that carried the GlcCerase mutations F213I/F213I, L444P/L444P, and N370S/N370S, respectively, were used.40 The culture medium was replaced every 2 d with fresh medium. For enzyme activity enhancement assay, cells were cultured in the presence of different concentrations of the pharmacological chaperones 6S-NOI-NJ and 6S-NAdB-NJ or their corresponding 1:1 complexes with the mannosylated carrier (ManS)3-βCD for 5 days and harvested by scraping. Cytotoxicity of the compounds was monitored by measuring the lactate dehydrogenase activities in the cultured supernatants (LDH assay kit, Wako, Tokyo, Japan).

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presence of different concentrations of the pharmacological chaperones 6S-NOI-NJ and 6S-NAdB-NJ or their corresponding 1 : 1 complexes with the mannosylated carrier (ManS)3-βCD for 5 days and harvested by scraping. Cytotoxicity of the compounds was monitored by measuring the lactate dehydrogenase activities in the cultured supernatants (LDH assay kit, Wako, Tokyo, Japan).

Immunofluorescence. All the procedures were carried out at room temperature as described previously.[¡Error! Marcador no definido.] Cells on the coverslips were fixed with 4% paraformaldehyde in phosphate buffered saline (PBS) for 30 min. Cells or brain sections were permeabilized with 0.25% Triton X-100 in PBS for 15 min, blocked with 1% bovine serum albumin (BSA) for 1 h, and incubated with primary antibodies for 1 h. Bound antibodies were detected with Alexa-Fluor-conjugated secondary antibodies for 1 h. Samples were mounted on slides with mounting media (Vector Laboratories, Burlingame, CA, USA) and fluorescence images were obtained sequentially using a confocal laser microscopy (Leica TSC SP-2; Wetzler, Germany). Fluorescence intensity was measured using Leica confocal software.

Enzyme-linked lectin assays (ELLA). Nunc-Inmuno plates (MaxiSorp™) were coated overnight with yeast mannan at 100 μL per well diluted from a stock solution of 10 μg mL^{-1} in 10 mM phosphate buffer saline (PBS, pH 7.3 containing 0.1 mM Ca^{2+} and 0.1 mM Mn^{2+}) at rt. The wells were then washed three times with 300 μL of washing buffer (containing 0.05% (v/v) Tween 20) (PBST). The washing procedure was repeated after each of the incubations throughout the assay. The wells were then blocked with 150 μL per well of 1% BSA/PBS for 1 h at 37 °C.

For determination of Con A binding affinity, the wells were filled with 100 μL of serial dilutions of peroxidase labeled Con A from 10−1 to 10−5 mg mL−1 in PBS, and incubated at 37 °C for 1 h. The plates were washed and 50 μL per well of 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) (0.25 mg mL−1) in

citrate buffer (0.2 M, pH 4.0 with 0.015% H_2O_2) was added. The reaction was stopped after 20 min by adding 50 μL per well of 1 M H_2SO_4 and the absorbances were measured at 415 nm. Blank wells contained citrate-phosphate buffer. The concentration of lectin that displayed an absorbance between 0.8 and 1.0 was used for inhibition experiments. In order to carry out the inhibition experiments, each inhibitor was added in a serial of 2-fold dilutions (60 μL per well) in PBS with 60 μL of the desired lectin–peroxidase conjugate concentration on Nunclon™ (Delta) microtiter plates and incubated for 1 h at 37 °C. The above solutions (100 μL) were then transferred to the mannancoated microplates, which were incubated for 1 h at 37 °C. The plates were washed and the ABTS substrate was added (50 μL per well). Color development was stopped after 20 min and the absorbances wer measured.

For determination of recombinant human MMR (rhMMR) binding affinity, the wells were filled with 100 μL of serial dilutions of rhMMR from a 10 mg mL−1 stock solution in PBS (pH 7.3 containing 0.1 mM Ca^{2+} and 0.1 mM Mn^{2+}), and incubated at 37 °C for 1 h. The plates were washed three times with PBST as described above and 100 μL of a solution of biotinylated anti-human MMR antibody (0.2 mg mL^{-1}; R&D Systems) in PBS was added in each well, and the plates were further incubated for 1 h at 37 °C. The complex NeutrAvidin®-biotinylated HRP was preformed separately by successively adding to Tris buffer (9.6 mL, 50 mM, pH 7.6) a solution of NeutrAvidin® (100 μg mL^{-1} in Tris buffer, 1.2 mL; Thermo Scientific) and a solution of biotin-conjugated HRP (25 μg mL^{-1} in Tris buffer, 1.2 mL; Thermo Scientific). The mixture was shaken for 30 min at rt and the solution was immediately transferred into the plates (60 μL per well). After 1 h at 37 °C, these plates were washed twice with Tris (250 μL per well) and ABTS (0.25 mg mL^{-1}, 50 μL per well) in citrate buffer (0.2 M, pH 4.0 with 0.015% H_2O_2) was added. After 5 min at rt, the optical density was measured at 415 nm. Blank wells were processed with anti-human MMR antibody as well as NeutrAvidin®-biotinylated HRP. The concentration of rhMMR that displayed a absorbance between 0.8 and 1.0 was used for inhibition experiments. For the competitive lectin binding inhibition experiment,

(ManS)3-βCD was mixed in a serial of 2-fold dilutions (60 mL per well) in HEPES buffer (20 mM, pH 7.4) with 60 mL of the appropriate rhMMR concentration in PBS buffer on Nunclon® (Delta) microtitre plates and incubated for 1 h at 37 °C. The above solutions (100 μL) were then transferred to the mannan-coated titer plates, which were incubated for 1 h at 37 °C. The plates were washed and the solution of biotinylated anti-human MMR antibody in PBS (100 μL) was added in each well, and the plates were further incubated for 1 h at 37 °C. Then the NeutrAvidin® solution was transferred into the plates (60 μL per well). After 1 h at 37 °C, these plates were washed twice with Tris (250 μL per well) and ABTS was added (50 μL per well). Optical density at 415 nm was determined after 5 min.

Results in triplicate were used for plotting the inhibition curves for each individual ELLA experiment. Typically, the IC50 values (concentration required to achieve 50% inhibition of the lectin association to the coating polysaccharide) obtained from several independently performed tests were in the range of ±15%. Nevertheless, the relative inhibition values calculated from independent series of data were highly reproducible. The inhibition for methyl α-D-gluco- and mannopyranoside were included as positive and negative controls, respectively.

Determination of mice macrophage adhesion. For evaluation of the interaction with macrophages, the procedure reported by Muller and Schuber for mannosylated liposomes was adapted. Briefly, resident peritoneal macrophages were obtained from female Balb/c mice (6 to 8 weeks old) and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% decomplemented fetal calf serum (FCS) containing heparin (5 U mL^{-1}). The cell number was adjusted to 106 cells mL^{-1} and the suspension was plated (final volume 1 mL) in multiwell plates. After 2 h under a humidified atmosphere of 5% CO2 in air (final pH 7.4), nonadherent cells were eliminated by rinsing the dishes three times with PBS. The adherent cells, 24 h after their isolation, were fed with fresh serum-less DMEM and incubated with different amounts of TNS: $(ManS)_3$-βCD complex. TNS :

βCD complex was used as control. After the incubation time, the medium was pipetted-off and the cells washed four times with cold PBS (4 °C). TNS associated with the cells was determined fluorimetrically (JASCO fluorimeter, model FP-715, excitation at 308 nm and monitoring emission at 443 nm) after cell digestion in 1 mL PBS containing 0.1% of emulphogene BC-720, and scraped with a rubber policeman. Standard fluorescence curves were establishe under the same conditions with aliquots of the initial TNS: $(ManS)_3$-βCD and TNS: βCD preparations in order to correlate the measured fluorescence intensity with the amount of CD derivative. Experiments were performed in duplicate, an results did not differ more than 5%.

Human macrophage internalization monitoring by fluorescence microscopy. THP-1 human monocytic cells were cultured in RPMI medium supplemented with penicillin, streptomycin, and 10% fetal bovine serum at 37 °C in a humidified 5% CO2 atmosphere. Cells were seeded onto 6-well plates at 1.5 × 3 106 cells per well. THP-1 monocytic cells were differentiated into macrophage- like cells by phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich) incubation at a final concentration of 100 ng mL^{-1} for 3 d and it was followed by 1 d in PMA-free medium before treatments. THP-1 macrophages were grown on 1 mm (Goldseal No. 1) glass coverslips for 24 h in RPMI containing 10% fetal bovine serum. After treatment, cells were rinsed once with PBS, fixed in 3.8% paraformaldehyde for 5 min, and permeabilized in 0.1% saponin for 5 min. For nuclei staining, glass coverslips were then rinsed with PBS for 3 min, incubated for 1 min with PBS containing Hoechst 33 342 (1 mg mL^{-1}) and washed with PBS (three 5 min washes). Finally, the coverslips were mounted onto microscope slides using Vectashield Mounting Medium (Vector Laboratories, Burlingame, CA, USA). 3D projections were performed using a Delta-Vision system (Applied Precision; Issaquah, WA) with an Olympus IX-71 microscope (Shinjuku, Japan) with 100× objective/1.35 NA and filters set for DAPI, fluorescein isothiocyanate provided by Applied Precision. Acquired z planes were separated by 0.3 μm, and an average of 50 planes was taken for each cell.

The 3D stacks were subjected to Quick Projection using the Softworx software. This allows confirming that the fluorescence is located inside the cell and not just in the cell membrane. Quantification of fluorescence signal was performed in 200 cells using ImageJ software (National Institutes of Health, Bethesda, Maryland, USA).

Vesicle preparation nanoparticles of amphiphilic derivatives.

Preliminary studies were performed in samples by weight in the compound into test tubes and adding water. The dispersion was sonicated in a water bath and then measured. The thin-film hydration method was used for further studies: An amount of the derivative was weighted in a round-bottomed flask and 1-2 mL of MeOH was added until the compound was dissolved completely. The solvent was evaporated by rotatory evaporation to obtain a homogeneous film. Water at 50 ºC was added and the film was hydrated 30 min at 50 ºC. The sample was the sonicated for 30 min.

Determination of CMC via pyrene fluorescence measurements.

In order to assess the amphiphilicity, the critical micelle concentrations (CMC) of some derivatives have been determined using an established fluorescence technique based on pyrene.[1] The onset of micelle formation can be observed in a shift of the fluorescence excitation spectra of the samples at an emission wavelength of 372 nm. In the concentration range of aqueous micellar solutions, a shift of the excitation band in the 335 nm region toward higher wavelengths confirms the incorporation of pyrene in the hydrophobic interior of micelles. The ratio of the fluorescence intensities at 339 and 335 nm was used to quantify the shift of the broad excitation band. The critical micelle concentrations were determined from the crossover point in the low concentration range. Fluorescence spectra were recorded with an F-2500 Hitachi spectrofluorophotometer and conventional 1-cm quartz cuvettes at 37 ± 0.1 ºC, using 2.5 mm excitation and emission slits.

General methods for transfection experiments. Polyethylenimine 25 (bPEI, MW 25 kDa, branched) was purchased from Aldrich. The plasmid pCMV-Luc VR1216 (6934 bp) encoding luciferase (Clontech, Palo Alto, CA, USA) used for transfection experiments was amplified in *E. coli*, isolated, and purified using Qiagen Plasmid Giga Kit (Qiagen GMBH, Hilden, Germany). The plasmid pCMV100-IL-12 (5500 bp) encoding interleukin-12 (IL-12) was kindly provided by Dr. Chen Qian, (University of Navarra). The following materials were used for DNAse I protection assays: agarose D-1 (Pronadisa, Madrid, Spain), Tris-boric acid-EDTA Buffer (10 x TBE Buffer) (Invitrogen, Barcelona, Spain), DNAse I and ethidium bromide (Gibco BRL, Barcelona, Spain). Sodium dodecyl sulphate (SDS) and NaCl (Roig Farma, Barcelona, Spain) were used to release DNA from the complexes. Ethylenediaminetetraacetic (EDTA) acid and DMSO Hibry-Max ® were supplied from Sigma. Alamar blue dye was purchased from Accumed International Companies (Westlake, OH, USA).

Cell culture. HepG2 (human hepatoblastoma) and COS-7 (American green monkey kidney) cells (American Type Culture Collection, Rockville, MD, USA) were maintained at 37 ºC under 5% CO_2 in complete medium constituted by Dulbecco's modified Eagle's medium-high glucose + glutaMAX® (Gibco BRL Life Technologies) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), penicillin (100 U/mL) and streptomycin (100 µg/mL). Cells were passaged by trypsinization twice a week.

Preparation of pDNA complexes and polyplexes. For *in vitro* assays, the quantities of compound used were calculated according to the desired DNA concentration of 5 µg/mL (15 µM phosphate), the molecular weight and the number of protonable nitrogens in the selected CD derivative or cationic polymer (bPEI, 25 kDa).pDNA complexes and polyplexes were prepared with plasmid DNA and the corresponding gemini-type polycationic suoramolecule and bPEI respectively, at N/P (atomic ratio) 5 and 10. Concerning the preparation of the DNA complexes, DNA was diluted in BHG (HEPES

10 mM, pH 7.4, glucose 5% w/v); then the desired amount of derivative was added from 1000 µM or 3000 µM stock solution in DMSO in order to achieve the desired concentrations of the amphiphilic derivatives for a final N/P 5 ratio. For N/P 10 formulations, the concentrations of CD derivatives were double.

The preparation was orbitally stirred for 2 h and used for characterization or transfection experiments. For bPEI, a solution of bPEI 1 M (H_2O) was diluted in distilled water to a final concentration of 0.01 M. A solution of DNA (10 µg/mL) in BHG was mixed with the same volume of a bPEI solution containing the desired amount of polymer, to give a 5 µg/mL DNA solution. The preparation was briefly vortexed and kept at room temperature for 30 min.

For *in vivo* assays, the DNA concentration was set at 300 µg/mL. paCDs derivatives were added from a 16.6 mM stock solution (DMSO:sterile H_2O 1:2).

Agarose gel electrophoresis. Each vector:pDNA complex (20 µL, 0.4 µg of plasmid) was submitted to electrophoresis for about 30 min under 150 V through a 0.8% agarose gel in TAE 1X (Tris-acetate-EDTA) buffer and stained by spreading GelRed Nucleic Acid Stain (Biotium). The DNA was then visualized after photographing on an *Alphaimager Mini UV* transilluminator. The plasmid integrity in each sample was confirmed by electrophoresis after decomplexation with sodium dodecyl sulfate (SDS, 8%).

DNA condensation/ protection assays. 50 µL of CDplexes were prepared in water at N/P ratio 5 and 10 to a final concentration of 50 µg/mL. Then, samples were electrophoresed for 30 min under 150 mV in 0.8% agarose gel. For protection assays, DNAse I (1U/µg pDNA) was added to each sample and stirred for 30 min at 37 ºC. 20 µL of EDTA 0.25 M was added to inactivate DNAse and the sample was vortexed and incubated for 5 min. 20 µL of SDS 25% was added and further incubated for 5 min.

Samples were electrophoresed as described above. Plasmid integrity was compared with free pDNA treated and untreated.

Particle size and zeta-potential measurements. The size of the CDplexes was measured by dynamic light scattering (DLS), and the overall charge by "Mixed Mode Measurement" phase analysis light scattering (M3-PALS) measurements using a Zeta Nano Series (Malvern Instruments, Spain). All measurements were performed in HEPES 10 mM, 5% glucose, pH 7.4, in triplicate. Size results are given as volume distribution of the major population by the mean diameter with its standard deviation.

In vitro transfection activity. The procedure for *in vitro* transfection assays was the same for both cell lines. Cells were seeded in medium in 48-well plates (Iwaki Microplate, Japan), and incubated for 24 h at 37 ºC in 5% $CO_{2.}$ After this, the medium was removed and 0.3 mL of complete medium (without serum) or serum (activated FBS) and 0.2 mL of complexes (containing 1 µg of pDNA) were added to each well. After 4 h incubation the medium was replaced for complete medium and the cells were further incubated for 48 h. Cells were washed with phosphate-buffered saline (PBS) and lysed with 100 µL of Reporter Lysis Buffer (Promega, Madison, WI, USA) at room temperature for 10 min, followed by a freeze-thaw cycle. 20 µL of the supernatant was assayed for total luciferase activity using the luciferase assay reagent (Promega), according to the manufacturer's protocol. A luminometer (Sirius-2, Berthold Detection Systems, Innogenetics, Diagnóstica y Terapéutica, Barcelona, Spain) was used to measure luciferase activity. The protein content of the lysates was measured by de DC protein Assay Reagent (Bio-Rad, Hercules, CA, USA) using bovine serum albumin as the standard. The data were expressed as nanograms of luciferase (based on a standard curve for luciferase activity) per milligram of protein. Citokine levels were obtained using the kit BD OptEIA ELISA Set (Pharmingen, San Diego, CA, U.S.A.) for IL-12 p40 following the manufacturer's

instructions. Values were calculated based on a standard curve. Samples were analized in a plate spectrophotometer Power Wave XS and a data processor KC junior, BioTek®.

Cell viability. Cell viability was quantified by a modified Alamar blue® assay (Invitrogen). Briefly, 1 mL of 10% (v/v) Alamar blue dye in complete medium was added to each well 48 h post-transfection. After 2.5 h of incubation at 37 ºC, 200 µL of the supernatant was assayed by measuring the absorbance at 570 and 600 nm. Cell viability (as percentage of control cells) was calculated according to the formula (A_{570} - A_{600}) of treated cells x 100/(A_{570} - A_{600}) of control cells.

In vivo transfection activity. Female Balb-c mice (6-8 weeks of age, 20-25 grams weigh) were purchased from Harlan Ibérica Laboratories. All animals were studied in accordance with guidelines established by Directive 86/609/EEC and with the approval of the Committee on Animal Research at the University of Navarra (Pamplona, 033/00). Individual mice in groups of eight were injected via the tail vein with 200 µL of CDplexes containing 60 µg of pCMV-Luc at N/P 5 and 10. Naked DNA was injected as control. Twenty four hours after injection the mice were sacrificed. The liver, heart, lungs and spleen were collected and washed with cold PBS. The organs were homogenized with 1 mL lysis buffer using an homogenizer at 5000 rpm (Mini-Beadbeater; BioSpec Products, Inc., Bartlesville, OK, USA) and centrifuged at 10000 rpm for 3 min. 20 µL of the supernatant were analysed for luciferase activity following the same procedure as for *in vitro* assays.

Statistical Analysis. Statistical analyses were performed using SPSS software from SPSS Inc. (Chicago, IL, USA). The analysis of the transfection efficiency of CDplexes was performed with a two-tailed unpaired Student´s t-test. $P < 0.05$ was considered statistically significant.

Synthesis of dodecanethiol coated gold nanoparticles (DDT-Au NPs). A solution of tetrachloroaurate acid in milli-Q water (25 mL, 0.03 M) was mixed with a solution of tetraoctylammonium bromide in toluene (80 mL, 0.05 M). The two phases mixture was vigorously stirred until all the tetrachloroaurate was transferred into the organic layer, and the aqueous layer was discarded. To the solution was added dropwise a $NaBH_4$ aqueous solution (25 mL, 0.35 M) for 1 minute, then the mixture was stirred for 1 h. The biphasic system was washed with 0.01 M HCl (1 x 25 mL), 0.01 M NaOH (1 x 25 mL) and H_2O milli Q (3 x 25 mL). Aqueous layers were discarded and the organic phase was stirred overnight at rt. Dodecanethiol (10 mL, 42 mmol) was added and the mixture was refluxed for 3 h. The system was cooled to rt and spin-dried at 2000 rpm for 10 min. The supernatant was recovered, and MeOH was added to reach 1:1 mixture to precipitate the NPs and eliminate the excess of dodecanethiol, and the system was spin-dried for 10 min at 2000 rpm. Then supernatant was discarded and the precipitate was suspended in 1 mL of $CHCl_3$. The final concentration of DDT-Au NPs was determined by UV spectrometry. A small aliquot of NPs was 1000-fold diluted and absorbance was measured using a $\varepsilon = 8.63 \cdot 10^6\ m^{-1}cm^{-1}$.[50]

Biological assays with TLR4 receptors. Expression plasmid for mouse MD-2 was a gift from Dr. Y. Nagai (University of Tokyo, Japan). Expression plasmid for mouse TLR4 was purchased from InvivoGen (CA, USA). Expression plasmids containing sequences of human TLR4 and MD-2 as well as the pELAM-1 firefly luciferase plasmid were a gift from Dr. C. Kirschning (Technical University of Munich, Germany). The Renilla luciferase phRL-TK plasmid was purchased from Promega (WI, USA). The human embryonic kidney (HEK) 293 cells were provided by Dr. J. Chow (Eisai Research Institute, Andover, USA). HEK293 cells were grown in DMEM supplemented with 10 % FBS. Compounds were dissolved in 100% DMSO to provide 4 mM stock solutions; further working dilutions were prepared immediately before stimulation with cell medium (DMEM supplemented with 10 % FBS).

Cell activation assay–NF-kB-luciferase reporter assay. HEK 293 cells were seeded in 96-well Costar plates (Corning, NY, USA) at 1.6·104 cells/well and incubated overnight in a humidified atmosphere (5 % CO_2) at 37 °C. The next day, when cells were 40-60 % confluent, they were co-transfected with MD-2 (10 ng), NF-kB-dependent luciferase (70 ng) and constitutive Renilla (15 ng) reporter plasmids and TLR4 plasmid (1 ng) using PEI (7.5 molar polyethylenimine pH 7.5, Polysciences) transfection reagent. Cells were stimulated 4 hours after transfection with the synthetic compounds, then1 h later with LPS (5 nM) that was extensively vortexed immediately prior to stimulation. Cells were lysed after 16 hours of stimulation in 1x reporter assay lysis buffer (Promega, USA) and analyzed for reporter gene activities using a dual-luciferase reporter assay system. Relative luciferase activity (RLA) was calculated by normalizing each sample's firefly luciferase activity for constitutive Renilla activity measured within the same sample. When plotting the data the value of the wild type MD-2·TLR4 sample stimulated with LPS was normalized to 100 and other values were adjusted accordingly.

HEK-Blue™ assay. HEK-Blue-TLR4 cells (InvivoGen) were cultured in DMEM high glucose medium supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 1x Normocin (InvivoGen), 1x HEK-Blue Selection (InvivoGen). Cells were detached by the use of a cell scraper and the cell concentration was estimated by using Trypan Blue (Sigma Aldrich). The cells were diluted in DMEM high glucose medium supplemented as described before and seeded in multiwell plate at a density of $2x10^4$ cells/well in 200 µL. After overnight incubation (37°C, 5% CO_2, 95% humidity), supernatant was removed, cell monolayers were washed with warm PBS without Ca^{2+} and Mg^{2+} and treated with increasing concentrations of compounds dissolved in DMSO-EtOH (1:1). After 30 minutes, the cells were stimulated with 10 nM LPS from *E. coli* O55:B5 (Sigma Aldrich) and incubated overnight at 37°C, 5% CO_2 and 95% humidity. As a control, the cells were treated with or without LPS (10 nM) alone. Then, the

supernatants were collected and 50 µL of each sample was added to 100 µL PBS, pH 8, 0.84 mM *p*-nitrophenylphosphate (pNPP) for a final concentration of 0.8 mM pNPP. Plates were incubated for 2-4 h in the dark at rt and then the plate reading was assessed by using a spectrophotometer at 405 nm (LT 4000, Labtech). The results were normalized with positive control (LPS alone) and expressed as the mean of percentage ± SD of at least three independent experiments.

MTT cell viability assay. HEK-Blue cells were seeded in 100 µL DMEM without Phenol Red at a density of $2x10^4$ cells per well. After overnight incubation, 10 µL compounds were added and the plates were incubated overnight at 37 °C, 5% CO_2, 95% humidity. DMSO and PBS were included as control. Then 10 µl of MTT solution (5 mg/mL in PBS) were added to each well. After 3 h incubation (37 °C, 5% CO_2, 95% humidity), HCl 0.1 N in isopropanol was added (100 µl/well) to dissolve formazan crystals. Formazan concentration in the wells was determined measuring the absorbance at 570 nm (LT 4000, Labtech). The results were normalized with untreated control (PBS) and expressed as the mean of percentage ± SD of three independent experiments.

***In vivo* endotoxin inhibition.** C57BL/6J mice (11-13 weeks old) were randomly assigned into groups and injected intraperitoneally with vehicle control (5 % DMSO in PBS) (groups none and LPS only) or the inhibitory compound ($2x10^{-7}$ mol compound/mouse for compounds **5-11**, all in 5 % DMSO solution). One hour later the mice were injected intraperitoneally with vehicle control (PBS) (group none) or with LPS from *E. coli* 055:B5 ($1x10^{-9}$ mol / mouse ≈ 10 µg LPS / mouse). Three hours later the blood was collected. Serum was tested with the mouse TNF-α ELISA kit ("ReadySetGo", eBioscience) to determine the levels of mouse TNF-α. The experiment was performed according to the manufacturer's instructions.

General Methods in Homogeneous Synthesis.

Acetylation was carried out by dissolving the compound in Ac_2O-py (1:1, 10 mL/g) at 0 ºC. The reaction mixture was stirred at room temperature, then poured into water/ice and extracted with CH_2Cl_2. Organic layer was washed with 2 N H_2SO_4 and satured aqueous solution of $NaHCO_3$, then dried ($MgSO_4$) and concentrated. The resulting residue was purified by column chromatography.

Deacetylation was carried out using Zemplen procedure.[2] Addition f 1 M MeONa (0.1 equiv./Ac mol) in MeOH at rt, followed by neutralization with solid CO_2, evaporation of the solvent and purification by column chromatography in some cases.

Trityl cleavage was achieved by treatment with $BF_3 \cdot Et_2O$ or PTSA and MeOH in DCM. The mixture was stirred, under Ar atmosphere, at room temperature for 2 h. H_2O was added and the organic layer was extracted and washed with satured aqueous solution of $NaHCO_3$, dried ($MgSO_4$), filtered and concentrated.

Boc cleavage was achieved by treatment with 1:1 CH_2Cl_2-TFA or 1:1 H_2O-TFA at rt for 2 h. Then, the solvent was eliminated under reduced pressure and coevaporated several times with water. The residue was submitted to column chromatography in most cases and the pure compound was dissolved in a HCl diluted solution and freeze-dried to yield the product as hydrochloride.

CuAAC reaction was carried out in a microwave reactor vial, alkyne, azide, $CuSO_4 \cdot 5H_2O$ (0.1 equiv. per alkyne), and sodium ascorbate (0.2 equiv. per alkyne) in DMF-H_2O were successively added. The resulting suspension was heated under microwave irradiation at 80 °C for 30 min., water was added and the aqueous phase was extracted with EtOAc. The organic phases were combined, dried (Na_2SO_4) and

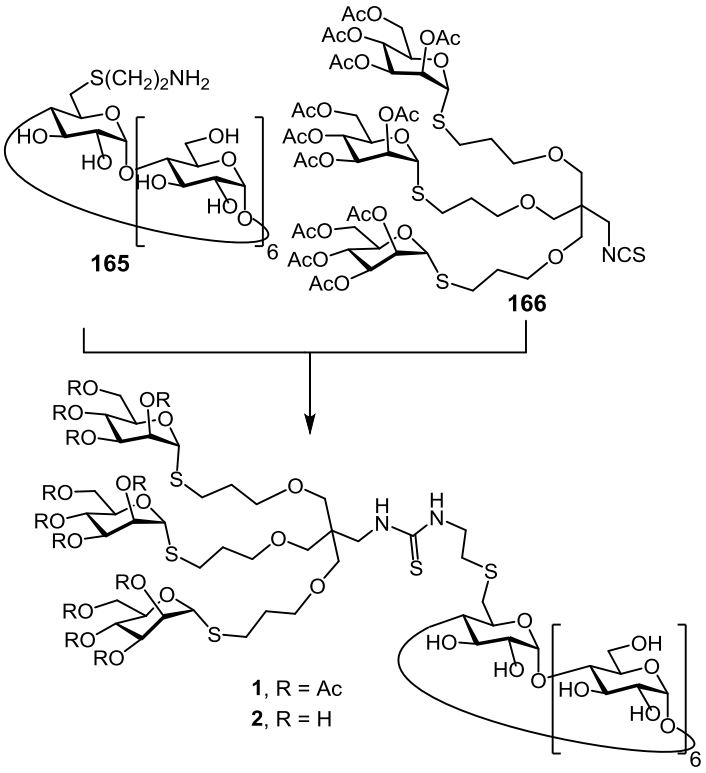
concentrated under reduced pressure. Traces of copper salts were removed by filtration under silica gel eluting with CH_3CN-H_2O-NH_4OH (15:0.5:0.5) and the residue was then purified by column chromatography.

9.2. Starting Materials.

- 2,2,2-Tris[5-(2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosylthio)-2-oxapentyl]ethyl isothiocyanate (**166**).[3]
- 2-[*N*,*N*-Bis(2-(*N*-*tert*-butoxyaminocarbonyl)ethylamino]ethyl isothiocyanate (**167**).[4]
- *tert*-Butyl *N*-(2-isothiocyanatoethyl) carbamato (**168**).[5]
- 3-Bis[2-*tert*-butoxycarbonylamino)ethyl]propargylamine (**169**).[6]
- Methyl 6-*O*-trityl-α-D-glucopyranoside (**171**).[7]
- Methyl 6-azido-6-deoxy-α-D-glucopyranoside (**172**).[8]
- 6,6'-Di-*O*-trityl-α,α'-trehalose (**173**).[9]
- 6,6'-Dideoxy-6,6'-diiodo-α,α'-trehalose (**176**).[10]
- 2,2,2-Tris[5-(2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosylthio)-2-oxapentyl]ethyl azide (**197**).[¡Error! Marcador no definido.]
- *N*-*tert*-Butoxycarbonyl-4,7,10-trioxa-1,13-tridecanediamine (**198**).[11]
- Methyl 6-deoxy-6-iodo-α-D-glucopyranoside (**199**).[12]
- *N'*-Octyl-iminomethylidene-6-thionojirimycin (**6S-NOI-NJ**).[13]
- *N'*-[4-(Adamant-1-ylcarboxamido)butyl]iminomethylidene-6-thionojirimycin (**6S-NAdB-NJ**).[13]
- Methyl 4,6-*O*-(4-methoxybenzylidene)-α-D-glucopyranoside.[14]

9.3. New Compounds.

Preparation of biorecognizable cyclodextrins for targeted transport of pharmacological chaperones.



6^{I}-[2-[*N*'-[2,2,2-Tris[5-(2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosylthio)-2-oxapentyl]ethyl]thioureido]ethylthio]cyclomaltoheptaose (1). To a solution of **165** (94 mg, 72 μmol) and Et_3N (20 μL, 144 μmol) in dry DMF (1 mL), a solution of **166** in dry DMF (1 mL) was added. The reaction mixture was stirred at rt for 72 h preserving the pH at 8-9 with Et_3N. The reaction mixture was then concentrated and the residue was purified by column chromatography (MeCN → 3:1 MeCN-H_2O). Yield: 93 mg (50%). R_f = 0.66 (6:3:1 MeCN-H_2O-NH_4OH); $[\alpha]_D$ = +57.8 (c 1.0, MeOH).

^{1}H NMR (500 MHz, CD_3OD, 313 K): δ = 5.39 (bs, 3 H, H-1_{Man}), 5.36 (d, 3 H, $J_{1,2}$ =

1.6 Hz, $J_{2,3}$ = 2.8 Hz, H-2_{Man}), 5.30 (t, 3 H, $J_{3,4}$ = $J_{4,5}$ = 9.8 Hz, H-4_{Man}), 5.23 (dd, 3 H, H-3_{Man}), 5.04 (d, 1 H, $J_{1,2}$ = 3.7 Hz, H-1^{II}), 5.01-4.98 (m, 5 H, H-$1^{III-VII}$), 5.00 (d, 1 H, $J_{1,2}$ = 3.3 Hz, H-1^{I}), 4.41 (ddd, 3 H, $J_{5,6a}$ = 4.9 Hz, $J_{5,6b}$ = 2.1 Hz, H-5_{Man}), 4.30 (dd, 3 H, $J_{6a,6b}$ = 12.1 Hz, $J_{5,6a}$ = 5.2 Hz, H-$6a_{Man}$), 4.16 (dd, 3 H, $J_{5,6b}$ = 5.2 Hz, H-$6b_{Man}$), 3.97 (ddd, 1 H, $J_{4,5}$ = 9.3 Hz, $J_{5,6a}$ = 2.3 Hz, $J_{5,6b}$ = 6.6 Hz, H-5^{I}), 3.92-3.84 (m, 17 H, H-$3^{III-VII}$, H-6^{II-VII}), 3.88 (t, 1 H, $J_{3,4}$ = 9.5 Hz, H-3^{II}), 3.87 (t, 1 H, $J_{2,3}$ = $J_{3,4}$ = 9.3 Hz, H-3^{I}), 3.81-3.76 (m, 6 H, H-5^{II-VII}), 3.73 (m, 4 H, CH_2N, CH_2NH_{Cyst}), 3.58 (2d, 6 H, $^3J_{H,H}$ = 6.0 Hz, H-3_{Pent}), 3.54 (dd, 1 H, H-2^{I}), 3.54 (dd, 1 H, $J_{1,2}$ = 9.5 Hz, H-2^{II}), 3.56-5.49 (m, 11 H, H-$2^{III-VII}$, H-4^{II-VII}), 3.53 (m, 1 H, H-4^{I}), 3.47 (m, 8 H, CH_2NH, H-1_{Pent}), 3.18 (dd, 1 H, $J_{6a,6b}$ = 12.8 Hz, H-$6a^{I}$), 2.94 (dd, 1 H, H-$6b^{I}$), 2.88 (t, 2 H, $^3J_{H,H}$ = 7.2 Hz, CH_2S_{Cyst}), 2.87-2.78 (m, 6 H, H-5_{Pent}), 2.17-1.98 (4 s, 36 H, MeCO), 1.96 (m, 6 H, H-4_{Pent}).

^{13}C NMR (125.7 MHz, CD_3OD, 313 K): δ = 182.0 (CS), 170.9-170.1 (CO), 102.5-102.2 (C-1^{I-VII}), 84.6 (C-4^{I}), 82.6 (C-1_{Man}), 81.8-81.7 (C-4^{II-VII}), 73.4-73.2 (C-3^{I-VII}), 72.9 (C-5^{I-VII}), 72.5-72.3 (C-2^{I-VII}), 71.1 (C-2_{Man}), 69.8 (C-3_{Man}), 69.5 (C-3_{Pent}), 69.1 (C-5_{Man}), 66.3 (C-4_{Man}), 62.4 (C-6_{Man}), 60.8-60.6 (C-6^{II-VII}, C-1_{Pent}), 44.3 (CH_2N, CH_2N_{Cyst}), 33.3 (C-6^{I}), 32.1 (CH_2S_{Cyst}), 29.3 (C-4_{Pent}), 28.0 (C-5_{Pent}), 19.4-19.1 (*Me*CO).

ESIMS: *m/z* 1314.8 $[M + 2 Na]^{2+}$. Anal. Calcd for $C_{101}H_{158}N_2O_{64}S_5$: C, 46.93; H, 6.16; N, 1.08; S, 6.20; found: C, 47.11; H, 6.34; N, 0.89; S, 5.87.

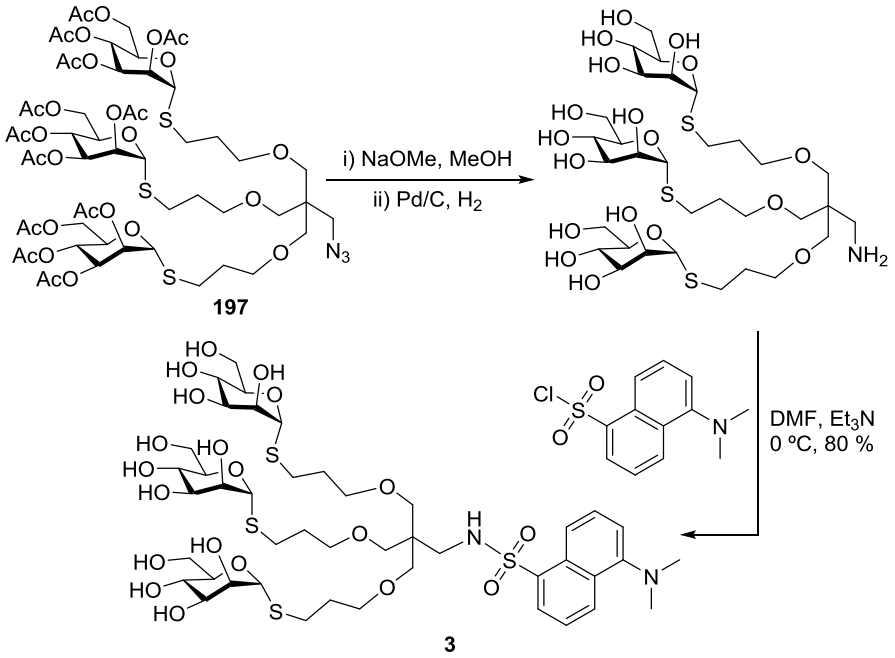
6^{I}-[2-[*N*'-[2,2,2-Tris[5-α-D-mannopyranosylthio)-2-oxapentyl]ethyl]-thioureido]ethylthio]cyclomaltoheptaose (2). The trivalent mannosyl carrier **2** was obtained by treating a solution of compound **1** (59 mg, 23 μmol) in dry MeOH (3 mL) with methanolic NaOMe (1 M, 27 μL, 0.23 μmol) at rt for 1 h. The reaction mixture was neutralized with Amberlite IR-120 (H^+) ion exchange resin, the resin filtered-off and the solvent evaporated under reduced pressure. Yield 46 mg (97%). $[\alpha]_D$ = +27.5 (c 1.0, H_2O); UV (H_2O): λ_{max} = 262 nm (ε_{mm} = 2308).

^{1}H NMR (500 MHz, D_2O, 323 K): δ = 5.53 (bs, 3 H, H-1_{Man}), 5.33 (m, 6 H, H-1^{II-VII}),

5.29 (d, 1 H, $J_{1,2}$ = 3.0 Hz, H-1^{I}), 4.29 (bs, 3 H, H-2$_{Man}$), 4.19-3.99 (m, 31 H, H-3^{II-VII}, H-5^{II-VII}, H-6^{II-VII}, H-1$_{Pent}$, H-3$_{Man}$, H-4$_{Man}$, H-5$_{Man}$, H-6$_{Man}$), 4.10 (t, 1 H, $J_{2,3}$ = $J_{3,4}$ = 9.1 Hz, H-3^{I}), 4.09 (m, 1 H, H-5^{I}), 3.97-3.85 (m, 12 H, H-2^{II-VII}, H-4^{II-VII}), 3.93 (m, 2 H, CH_2N_{Cyst}), 3.90 (dd, 1 H, H-2^{I}), 3.76 (t, 6 H, $^{3}J_{H,H}$ = 5.4 Hz, H-3$_{Pent}$), 3.75 (t, 1 H, H-4^{I}), 3.65 (s, 2 H, CH_2NH), 3.45 (bd, 1 H, $J_{6a,6b}$ = 11.2 Hz, H-6a^{I}), 3.15 (t, 2 H, $^{3}J_{H,H}$ = 6.5 Hz, CH_2S_{Cyst}), 3.07 (dd, 1 H, $J_{5,6b}$ = 7.3 Hz, H-6b^{I}), 2.99-2.87 (m, 6 H, H-5$_{Pent}$), 2.14 (m, 6 H, H-4$_{Pent}$).

^{13}C NMR (125.7 MHz, D_2O, 323 K): δ = 182.3 (CS), 102.6-102.1 (C-1^{I-VII}), 85.3 (C-1$_{Man}$), 85.0 (C-4^{I}), 81.3-80.9 (C-4^{II-VII}), 73.6-71.4 (C-2$_{Man}$, C-3$_{Man}$, C-5$_{Man}$, C-2^{I-VII}, C-3^{I-VII}, C-5^{I-VII}), 70.4 (C-3$_{Pent}$), 67.2 (C-4$_{Man}$), 61.5 (C-1$_{Pent}$), 60.5-60.2 (C-6^{II-VII}), 44.7 (CH_2N, CH_2N_{Cyst}), 34.2 (C-6^{I}), 33.8 (CH_2S_{Cyst}), 29.3 (C-4$_{Pent}$), 28.0 (C-5$_{Pent}$).

ESIMS: *m/z* 1062.3 $[M + 2 Na]^{2+}$. Anal. Calcd for $C_{77}H_{134}N_2O_{52}S_5$: C, 44.46; H, 6.49; N, 1.35; S, 7.71. Found: C, 44.25, H, 6.22; N, 1.03; S, 7.42.



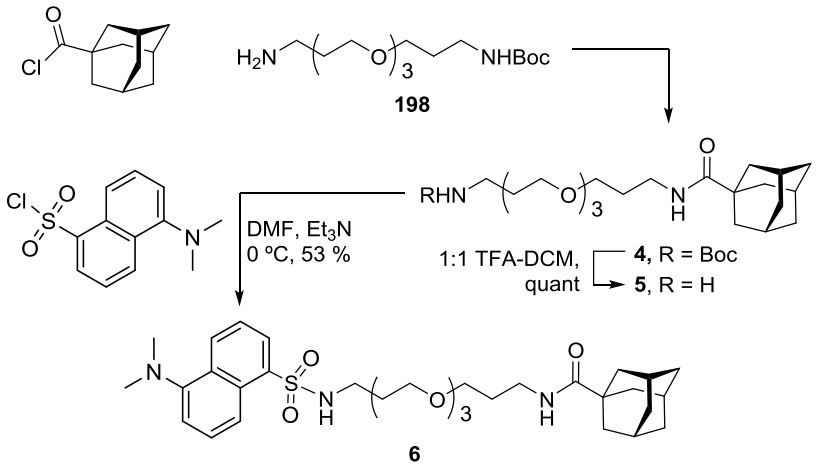
197 **3**

***N*-dansyl-2,2,2-tris[5-(α-D-mannopyranosylthio)-2-oxapentyl]ethyl amine (3)**. A solution of 2,2,2-tris[5-(2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosylthio)-2-oxapentyl]ethyl azide **197** (106 mg, 77 μmol) in MeOH (5 mL) was treated with a NaOMe (10% eq, 1 M methanolic solution, 92 μL) for 3 h. The solution was stirred at rt for 30 min, and then neutralised using Amberlite IR-120 (H^+) ion exchange resin, filtered and concentrated to dryness. The resulting deacetylated azide was then hydrogenated by treatment with 10% Pd/C (20 mg) under a H_2 atmosphere (1 atm) for 16 h. The reaction mixture was filtered and the solvents evaporated. The crude amine thus obtained was dissolved in anhydrous DMF (2.3 mL) and the solution was cooled to 0 °C. Et_3N (17 μL, 0.12 mmol) and 5-dimethylaminonaphtalene-1-sulfonyl chloride (32 mg, 0.12 mmol) were added and the reaction mixture was stirred for 24 h. at rt. The solvent was subsequently removed under reduced pressure and the residue was purified by column chromatography using 50:10:1 → 50:10:1 DCM-MeOH-H_2O as eluent. Yield: 66 mg (80% over three steps). $[\alpha]_D$ = +97.4 (*c* 0.7, MeOH); R_f = 0.33 (30:10:1 DCM-MeOH-H_2O); IR: ν_{max} = 3351, 1648, 1456, 1067 cm^{-1}.

^{1}H NMR (500 MHz, CD_3OD): δ = 8.61 (bd, $J_{3,4}$ = 8.5 Hz, 1 H, H-4_{Naph}), 8.34 (d, $J_{8,9}$ = 8.7 Hz, 1 H, H-9_{Naph}), 8.22 (dd, $J_{2,3}$ = 7.3 Hz, $J_{2,4}$ = 2.0 Hz, 1 H, H-2_{Naph}), 7.65 (m, 2 H, H-3_{Naph}, H-8_{Naph}), 7.32 (d, $J_{7,8}$ = 7.6 Hz, 1 H, H-7_{Naph}), 5.24 (bs, 3 H, H-1_{Man}), 3.95 (bd, 3 H, $J_{1,2}$ = 0.9 Hz, H-2_{Man}), 3.91 (m, 3 H, H-5_{Man}), 3.84 (dd, 3 H, $J_{6a,6b}$ = 11.9 Hz, $J_{5,6a}$ = 2.2 Hz, H-6a_{Man}), 3.78 (dd, 3 H, $J_{5,6b}$ = 5.5 Hz, H-6b_{Man}), 3.70 (m, 6 H, H-3_{Man}, H-4_{Man}), 3.30 (t, 6 H, $^3J_{H,H}$ = 5.9 Hz, H-3_{Pent}), 3.15 (bs, 6 H, H-1_{Pent}), 2.94 (m, 2 H, CH_2NH), 2.92 (s, 6 H, CH_3), 2.65 (m, 6 H, H-1_{Pent}), 1.78 (t, 6 H, H-4_{Pent}).

^{13}C NMR (125.7 MHz, CD_3OD): δ = 153.3 (C-6_{Naph}), 136.1 (C-1_{Naph}), 131.4-130.7 (C-2_{Naph}, C-4_{Naph}, C-5_{Naph}, C-10_{Naph}), 129.4 (C-8_{Naph}), 124.4 (C-3_{Naph}), 120.2 (C-9_{Naph}), 116.5 (C-7_{Naph}), 86.5 (C-1_{Man}), 74.9 (C-5_{Man}), 73.7 (C-2_{Man}), 73.1 (C-3_{Man}), 71.2 (C-3_{Pent}), 70.6 (C-1_{Pent}), 68.7 (C-4_{Man}), 62.7 (C-6_{Man}), 47.8 (CH_3), 45.9 (CH_2NH), 44.9 (C_q) 30.6 (C-4_{Pent}), 28.8 (C-5_{Pent}).

ESIMS: *m/z* 1099 $[M + Na]^+$; Anal. Calcd for $C_{44}H_{72}N_2O_{20}S_4$: C, 49.05; H, 6.74; N, 2.60; S, 11.91. Found: C, 48.67; H, 6.83; N, 2.34; S, 11.56.



***N*-(13-*tert*-butoxycarbonylamino-4,7,10-trioxa)tridecanyl-1-adamantanecarboxamide (4)**. To a stirred solution of commercial adamantane-1-carbonyl chloride (0.47 g, 2.38 mmol) in DCM (27 mL), a solution of *N*-*tert*-butoxycarbonyl-4,7,10-trioxa-1,13-tridecanediamine **198** (0.71 g, 2.23 mmol) and Et_3N (1.02 mL, 5.50 mmol) in DCM (27 mL) was added. The reaction mixture was stirred for 3 h at rt, and then diluted with DCM (30 mL), washed with H_2O (30 mL), an aqueous solution of 1 N HCl (30 mL) and saturated aqueous $NaHCO_3$ (35 mL), dried ($MgSO_4$), and concentrated. The resulting residue was purified by column chromatography (1:2 acetone-cyclohexane). Yield: 1.043 g (97%); R_f = 0.24 (1:2 acetone-cyclohexane); IR: ν_{max} = 3344, 2904, 1697, 1638, 1521, 1105 cm^{-1}.

^{1}H NMR (300 MHz, $CDCl_3$): δ = 6.28 (bs, 1 H, NHCO), 4.96 (bs, 1 H, NHBoc), 3.66-3.49 (m, 12 H, OCH_2), 3.33 (q, 2 H, $^3J_{H,H} = J_{NH,H}$ = 6.1 Hz, C*H*$_2$NHCO), 3.20 (q, 2 H, $^3J_{H,H} = J_{NH,H}$ = 6.1 Hz, C*H*$_2$NHBoc), 2.00 (bs, 3 H, CH), 1.81 (m, 6 H, CCH_2), 1.75 (m, 4 H, C*H*$_2$CH$_2$NH), 1.69 (m, 6 H, CH_2), 1.41 (s, 9 H, CMe_3).

^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 177.8 (CO amide), 155.9 (CO carbamate), 78.8 (*C*Me$_3$), 70.6, 70.5, 70.4, 70.3, 70.1, 69.5 (OCH_2), 40.4 (*C*H$_2$NHBoc), 39.2 (CH), 38.5

(*C*CO), 37.9 (*C*H_2NHCO), 36.5 (*C*CH_2), 29.6 (*C*$H_2$$CH_2$NHBoc), 28.9 (*C*$H_2$$CH_2$NHCO), 28.4 (C*Me*$_3$), 28.1 ($CH_2$).

ESIMS: *m/z* 505.3 $[M + Na]^+$; Anal. Calcd for $C_{26}H_{46}N_2O_6$: C, 64.70; H, 9.61; N, 5.80. Found: C, 64.84; H, 9.70; N, 5.72.

***N*-(13-amino-4,7,10-trioxa)tridecanyl-1-adamantanecarboxamide (5).** To a stirred solution of **4** (130 mg, 0.27 mmol) in DCM (5 mL), TFA (1 mL) was dropwise added. The reaction mixture was stirred at rt for 1 h, concentrated under reduced pressure, coevaporated several times with DCM and toluene, suspended in 10:1 water-HCl 0.1 N, freeze-dried, and neutralized with basic resin IRA67. Yield: 103 mg (quantitative); R_f = 0.40 (30:1:1 CH_3CN-H_2O-NH_4OH); IR: ν_{max} = 3350, 2902, 1634, 1525, 1098 cm^{-1}.

^{1}H NMR (300 MHz, $CDCl_3$): δ = 6.37 (bs, 1 H, NH), 3.55-3.46 (m, 12 H, OCH_2), 3.25 (q, 2 H, $^3J_{H,H} = J_{NH,H}$ = 6.3 Hz, C*H*$_2$NHCO), 2.84 (bs, 2 H, NH_2), 2.77 (bt, 2 H, $^3J_{H,H}$ = 6.3 Hz, C*H*$_2$$NH_2$), 1.94 (bs, 3 H, CH), 1.75 (bs, 6 H, CCH_2), 1.69 (m, 4 H, C*H*$_2$$CH_2$N), 1.62 (m, 6 H, CH_2).

^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 177.8 (CO), 70.3, 70.2, 70.1, 69.9, 69.3 (OCH_2), 40.3 (CH_2NH_2), 39.3 (*C*CO), 39.0 (CH), 37.7 (*C*H_2NHCO), 36.4 (*C*CH_2), 32.0(*C*$H_2$$CH_2NH_2$), 28.9 (*C*$H_2$$CH_2$NHCO), 28.0 ($CH_2$).

ESIMS: *m/z* 383.3 $[M + H]^+$. Anal. Calcd for $C_{21}H_{38}N_2O_4$: C, 65.93; H, 10.01; N, 7.32. Found: C, 65.79; H, 9.73; N, 6.98.

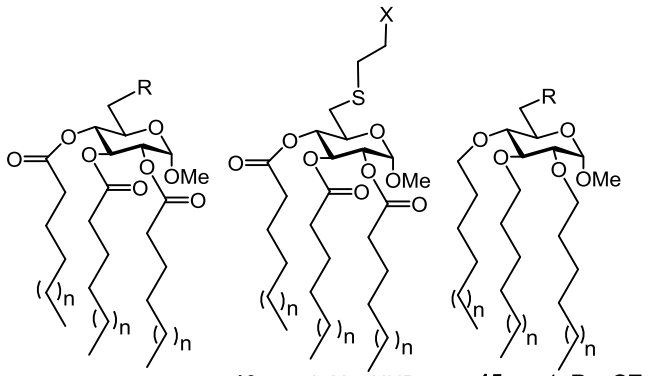
***N*-(13-dansylamino-4,7,10-trioxa)tridecanyl-1-adamantanecarboxamide (6).** A solution of **5** (263 mg, 0.687 mmol) in anhydrous DMF (15 mL) under Ar was cooled to 0 °C and Et_3N (92 μL, 0.742 mmol) and 5-dimethylaminonaphtalene-1-sulfonyl chloride (186 mg, 0.742 mmol) were added. The reaction was stirred for 4 h. Subsequently the solvent was removed under reduced pressure and the residue was purified by column chromatography (1:2 acetone-cyclohexane). Yield: 224 mg (53%); R_f = 0.32 (1:2 acetone-cyclohexane). IR: ν_{max} = 3282, 3056, 2906, 1635, 1525, 1425, 1095 cm^{-1}.

^{1}H NMR (300 MHz, $CDCl_3$): δ = 8.47 (d, $J_{3,4}$ = 8.6 Hz, 1 H, H-4_{Naph}), 8.28 (d, $J_{8,9}$ = 8.6 Hz, 1 H, H-9_{Naph}), 8.18 (dd, $J_{2,3}$ = 7.3 Hz, $J_{2,4}$ = 0.8 Hz, 1 H, H-2_{Naph}), 7.52-7.43 (m, 2 H, H-3_{Naph}, H-8_{Naph}), 7.12 (d, $J_{7,8}$ = 7.4 Hz, 1 H, H-7_{Naph}), 6.35 (bt, 1 H, NHCO), 5.99 (t, 1 H, $^{3}J_{H,H}$ = 5.9 Hz, $NHSO_2$), 3.59-3.46 (m, 12 H, OCH_2), 3.27 (q, 2 H, $^{3}J_{H,H}$ = $J_{NH,H}$ = 5.9 Hz, C*H*$_2$NHCO), 2.97 (q, 2 H, $J_{NH,H}$ = 5.9 Hz, C*H*$_2$NHSO$_2$), 2.82 (s, 6 H, CH_3), 1.91 (bs, 3 H, CH), 1.74, 1.73 (s, 6 H, CCH_2), 1.71-156 (m, 4 H, C*H*$_2$CH$_2$NH), 1.60 (m, 6 H, CH_2).

^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 177.0 (CO), 151.6 (C-6_{Naph}), 134.9 (C-1_{Naph}), 129.9-129.2 (C-2_{Naph}, C-4_{Naph}, C-5_{Naph}, C-10_{Naph}), 127.9 (C-8_{Naph}), 123.0 (C-3_{Naph}), 119.0 (C-9_{Naph}), 114.9 (C-7_{Naph}), 70.4, 70.2 (2 C), 70.0, 69.7, 69.5 (OCH_2), 45.2 (CH_3), 41.7 (CH_2NHSO_2), 40.2 (*C*CO), 38.9 (CH), 37.6 (*C*H$_2$NHCO), 36.3 (*C*CH$_2$), 28.8 (*C*H$_2$CH$_2$NHCO), 28.5 (*C*H$_2$CH$_2$NHSO$_2$), 27.9 (CH_2).

ESIMS: *m/z* 638.6 $[M + Na]^+$. Anal. Calcd for $C_{33}H_{49}N_3O_6S$: C, 64.36; H, 8.02; N, 6.82; S, 5.21. Found: C, 64.02; H, 7.90; N, 6.50; S, 4.88.

Preparation of cationic amphiphilic derivatives of glucose.



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8, n=9, R = **I**
9, n=1, R = N_3

10, n = 1, X = NHBoc
11, n = 1, X = NH_2·HCl
12, n = 9, X = NHBoc
13, n = 9, X = NH_2·HCl
14, n =1, X = N_3

15, n= 1, R = OTr
16, n= 1, R = OH
17, n= 9, R = OTr
18, n= 9, R = OH
19, n=1, R = **I**
20, n= 9, R = **I**
21, n=1, R= N_3
22, n=9, R = N_3

Methyl 6-deoxy-2,3,4-tri-*O*-hexanoyl-6-iodo-α-D-glucopyranoside (7). To a solution of methyl 6-deoxy-6-iodo-α-D-glucopyranoside **199** (3.13 g, 10.3 mmol) and DMAP (5.66 g, 45.9 mmol) in dry DMF (150 mL), hexanoic anhydride (14.3 mL, 61.8 mmol) was added dropwise at 0 ºC and the solution was stirred at rt, under Ar atmosphere, for 4 h. Then, MeOH (100 mL) was added and the mixture was stirred at rt for 2 h. The reaction mixture was poured onto ice-water (150 mL) and the aqueous solution was extracted with DCM (100 mL). The organic phase was then washed with 2 N H_2SO_4 (2 x 70 mL), H_2O (2 x 70 mL) and saturated aqueous $NaHCO_3$ (2 x 70 mL), dried ($MgSO_4$), filtered, concentrated and purified by column chromatography (1:12 EtOAc-petroleum ether). Yield: 3.30 g (55%). R_f = 0.55 (1:6 EtOAc-petroleum ether); $[\alpha]_D$ = +77.01 (*c* 1.0, DCM); IR: ν_{max} = 2956, 1747, cm^{-1}.

^{1}H NMR (300 MHz, $CDCl_3$): δ = 5.48 (t, 1 H, $J_{2,3} = J_{3,4}$ = 9.7 Hz, H-3), 4.95 (d, 1 H, $J_{1,2}$ = 3.8 Hz, H-1), 4.87 (m, 2 H, H-2, H-4), 3.78 (dt, 1 H, $J_{4,5}$ = 8.5 Hz, $J_{5,6a} = J_{5,6b}$ = 2.5 Hz, H-5), 3.46 (s, 3 H, OCH_3), (dd, 1 H, $J_{6a,6b}$ = 11.0 Hz, H-6a), 3.11 (dd, 1 H, H-6b), 2.25 (m, 6 H, H-2_{Hex}), 1.56 (m, 6 H, H-3_{Hex}), 1.56 (m, 12 H, H-4_{Hex}, H-5_{Hex}), 0.88 (m, 9 H, H-6_{Hex}).

^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 173-172 (CO), 96.7 (C-1), 72.13 (C-4), 70.8 (C-2), 69.1 (C-3), 68.2 (C-5), 55.72 (OMe), 34 (C-2_{Hex}), 31.2 (C-4_{Hex}), 24.4 (C-3_{Hex}), 22.2 (C-5_{Hex}), 13.8 (C-6_{Hex}), 3.67 (C-6).

ESIMS: *m/z* = 621.2 $[M + Na]^+$. Anal. Calcd for $C_{47}H_{78}I_2O_{15}$: C, 50.17; H, 7.24. Found: C, 50.24; H, 7.31.

Methyl 6-deoxy-6-iodo-2,3,4-tri-*O*-myristoyl-α-D-glucopyranoside (8). To a solution of methyl 6-deoxy-6-iodo-α-D-glucopyranoside **199** (300 mg, 0.98 mmol) in dry DMF (21 mL) and DMAP (722 mg, 5.9 mmol), myristic anhydride (2.6 g, 5.9 mmol) was added and the solution was stirred at rt, under Ar atmosphere, for 4 h. Solvent was evaporated, and MeOH-DCM (95:5, 25 mL) was added and the mixture was refluxed

until complete dissolution. The solution was concentrated and cold EtOH was added and the suspension was filtered off and dried. Yield: 623 mg (68%). R_f = 0.52 (1:8 EtOAc-cyclohexane); $[\alpha]_D$ = +59.5 (*c* 1.0, DCM); IR: ν_{max} = 1736 $cm^{-1.}$

^{1}H NMR (300 MHz, $CDCl_3$): δ = 5.48 (t, 1 H, $J_{2,3}$ = $J_{3,4}$ = 9.3 Hz, H-3), 4.95 (d, 1 H, $J_{1,2}$ = 3.7 Hz, H-1), 4.86 (t, 1 H, $J_{4,5}$ = 9.3 Hz, H-4), 4.85 (dd, 1 H, H-2), 3.78 (ddd, 1 H, $J_{5,6a}$ = 2.6 Hz, $J_{5,6b}$ = 8.6 Hz, H-5), 3.46 (s, 3 H, OCH_3), 3.26 (dd, 1 H, $J_{6a,6b}$ = 11.0 Hz, H-6a), 3.11 (dd, 1 H, H-6b), 2.37-2.15 (m, 6 H, CH_2CO), 1.55 (m, 6 H, C*H*$_2$$CH_2CO$), 1.24 (m, 60 H, CH_2), 0.88 (m, 9 H, H-6_{Hex}).

^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 172.9, 172.6, 172.4 (CO ester), 96.8 (C-1), 72.13 (C-4), 70.8 (C-2), 69.1 (C-3), 68.2 (C-5), 55.72 (OMe), 34.2, 31.2, 29.4, 29.3, 29.2, 29.1, 24.4, 22.2, 24.9, 24.8, 24.7, 22.7, 14.1 (CH_2), 14.1 (CH_3), 3.64 (C-6).

ESIMS: *m/z* = 957.5 $[M + Na]^+$, 1892 $[2M + Na]^+$. Anal. Calcd for $C_{49}H_{91}IO_8$: C, 62.93; H, 9.81. Found: C, 63.11; H, 9.89.

Methyl 6-azido-6-deoxy-2,3,4-tri-O-hexanoyl-α-D-glucopyranoside (9). To a solution of **7** (3 g, 5.01 mmol) in dry DMF (12 mL), NaN_3 (456 mg, 7 mmol,) was added. The reaction mixture was stirred overnight at 40 ºC, under Ar atmosphere. The mixture was poured into ice-water (20 mL), and the product was extracted with DCM (4 x 20 mL). The organic phase was dried ($MgSO_4$), filtered and concentrated. The residue was purified by column chromatography (1:12 EtOAc-cyclohexane). Yield: 2.13 g (83%). R_f = 0.50 (1:6 EtOAc-cyclohexane). $[\alpha]_D$ = +86.88 (*c* 1.0, DCM); IR: ν_{max} = 2014, 1747, 733 cm^{-1}.

^{1}H NMR (300 MHz, $CDCl_3$): δ = 5.48 (t, 1 H, $J_{2,3}$ = $J_{3,4}$ = 9.6 Hz, H-3), 4.98 (t, 1 H, $J_{4,5}$ = 9.6 Hz, H-4), 4.95 (d, 1 H, $J_{1,2}$ = 4.0 Hz, H-1), 4.86 (dd, 1 H, H-2), 3.95 (ddd, 1 H, $J_{5,6a}$ = 6.9 Hz, $J_{5,6b}$ = 2.7 Hz, H-5), 3.42 (s, 3 H, OCH_3), 3.32 (dd, 1 H, $J_{6a,6b}$ = 13.5 Hz, H-6a), 3.27 (dd, 1 H, H-6b), 2.40-2.15 (m, 6 H, H-2_{Hex}), 1.56 (m, 6 H, H-3_{Hex}), 1.27 (m, 12 H, H-4_{Hex}, H-5_{Hex}), 0.87 (t, 9 H, H-6_{Hex}).

^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 172.9, 172.6, 172.4 (CO), 96.8 (C-1), 70.7 (C-4), 69.5 (C-5), 69.4 (C-2), 68.7 (C-3), 55.5 (OMe), 51.1 (C-6), 34.1 (C-2_{Hex}), 31.2 (C-4_{Hex}), 24.1 (C-3_{Hex}), 22.1 (C-5_{Hex}), 13.8 (C-6_{Hex}).

ESIMS: *m/z* = 536.3 $[M + Na]^+$, 1049.6 $[2M + Na]^+$. Anal. Calcd for $C_{25}H_{43}N_3O_8$: C, 58.46; H, 8.44; N, 8.18. Found: C, 58.60; H, 8.42; N, 7.85.

Methyl 6-(2-*tert*-butoxycarbonylaminoethylthio)-2,3,4-tri-*O*-hexanoyl-α-D-glucopyranoside (10). To a solution of **7** (0.49 g, 0.83 mmol) in dry DMF (7.5 mL), Cs_2CO_3 (0.38 g, 0.16 mmol) and *N*-(2-mercaptoethyl) *tert*-butyl carbamate (196 μL, 1.16 mmol) were added, under Ar atmosphere, and the reaction mixture was stirred at 60 ºC for 48 h. The reaction mixture was concentrated and the crude product was dissolved in DCM (10 mL) and washed with H_2O (2 x 15 mL). The organic phase was dried ($MgSO_4$), filtered, concentrated, and the residue was purified by flash column chromatography (1:4 EtOAc-cyclohexane). Yield 0.45 g, (85%). R_f = 0.38 (1:3 EtOAc-cyclohexane); $[\alpha]_D$ = +62.1 (*c* 1.0, DCM); IR: ν_{max} = 2958, 1747, 1701cm^{-1}.

^{1}H NMR (300 MHz, $CDCl_3$): δ = 5.46 (t, 1 H, $J_{2,3} = J_{3,4}$= 10 Hz, H-3), 4.95 (t, 1 H, $J_{4,5}$ = 10.0 Hz, H-4), 3.41 (s, 3 H, OCH_3), 4.90 (d, 1 H, $J_{1,2}$ = 3.7 Hz, H-1), 4.83 (dd, 1 H, H-2), 3.90 (dt, 1 H, $J_{5,6b}$ = 8.0 Hz, H-5), 3.27 (q, 2 H, $J_{H,H}$ = 6.0 Hz, C*H*$_2$N), 2.81-2.50 (m, 4 H, C*H*$_2$S, H-6a, H-6b), 2.52 (m, 2 H, H-6a, H-6b), 2.31 (m, 6 H, H-2_{Hex},), 1.54 (m, 6 H, H-3_{Hex}), 1.41 (s, 9 H, CMe_3), 1.25 (m, 12 H, H-4_{Hex}, H-5_{Hex}), 0.86 (t, 9 H, H-6_{Hex}).

^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 172.9-172.0 (CO ester), 155.7 (CO carbamate) 96.7 (C-1), 71.5 (C-4), 70.9 (C-2), 69.3 (C-3), 69.9 (C-5), 55.1 (OMe), 39.58 (*C*H$_2$N), 33.7 (*C*H$_2$S), 33.0 (C-6), 34.0 (C-2_{Hex}), 24.5 (C-3_{Hex}), 28.2 (C*Me*$_3$), 22.1 (C-5_{Hex}), 13.6 (C-6_{Hex}).

ESIMS: *m/z* = 670.4 $[M + Na]^+$. Anal. Calcd for $C_{32}H_{57}NO_{10}S$: C, 59.32; H, 8.87; N, 2.16; S, 4.95. Found: C, 59.45; H, 8.93; N, 2.31; S, 4.71.

Methyl 6-(2-aminoethylthio)-2,3,4-tri-*O*-hexanoyl-α-D-glucopyranoside

hydrochloride (11). Compound **11** was obtained by treatment of **10** (0.29 g, 0.44 mmol) with 1:1 TFA-DCM (6 mL) and freeze-drying from 10:1 H_2O/0.1 N HCl solution. Yield: 0.27 g (quantitative). $[\alpha]_D$ = +72.0 (*c* 0.7, EtOAc); IR: ν_{max} = 2962, 1749 cm^{-1}.

^{1}H NMR (300 MHz, CD_3OD): δ = 8.05 (bs, 3 H, N*H*$_3$), 5.28 (t, 1 H $J_{2,3}$ = 9.7 Hz, $J_{3,4}$ = 9.7 Hz, H-3), 4.94 (t, 1 H, $J_{4,5}$ = 9.7 Hz, H-4), 4.88 (m, 1 H, $J_{1,2}$ = 3.8 Hz, H-1), 4.82 (dd, 1 H, H-2), 3.8 (m, 1 H, H-5), 3.34 (s, 1 H, OCH_3), 2.93 (bs, 2 H, C*H*$_2$N), 2.78 (m, 2 H, C*H*$_2$S), 2.73 (dd, 1 H, $J_{6a,6b}$ = 14.0 Hz, $J_{5,6a}$ = 2.7, H-6^a), 2.62 (dd, 1 H, $J_{5,6b}$ = 7.7 Hz, H-6b), 2.26 (m, 6 H, H-2$_{Hex}$), 1.44 (m, 6 H, H-3$_{Hex}$), 1.21 (m, 12 H, H-4$_{Hex}$, H-5$_{Hex}$), 0.82 (t, 9 H, H-6$_{Hex}$).

^{13}C NMR (75.5 MHz, CD_3OD): δ = 172-171 (CO), 95.98 (C-1), 71.2 (C-4), 70.6 (C-2), 69.4 (C-3), 69.1 (C-5), 55.1 (OMe), 39.1 (*C*H_2N), 32.5 (C-6), 30.5 (*C*H_2S), 33.1 (C-2$_{Hex}$), 31.1 (C-4$_{Hex}$), 24 (C-3$_{Hex}$), 22.1(C-2$_{Hex}$), 14.4 (C-6$_{Hex}$).

ESIMS: *m/z* = 548.3 $[M + H]^+$. Anal. Calcd for $C_{27}H_{50}ClNO_8S \cdot HCl$: C, 55.51; H, 8.63; N, 2.40; S, 5.49. Found: C, 55.37; H, 8.62; N, 2.26; S, 5.27.

Methyl 6-(2-*tert*-butoxycarbonylaminoethylthio)-2,3,4-tri-*O*-myristoil-α-D-glucopyranoside (12). To a solution of **8** (300 mg, 0.32 mmol) in dry DMF (5 mL), Cs_2CO_3 (147 mg, 0.45 mmol) and *tert*-butyl *N*-(2-mercaptoethyl)carbamate (76 μL, 0.45 mmol) were added, under Ar atmosphere, and the reaction mixture was stirred at 60 °C for 48 h. The reaction mixture was concentrated and the crude product was dissolved in DCM (10 mL) and washed with H_2O (2 x 15 mL). The organic phase was dried ($MgSO_4$), filtered and concentrated. The residue was purified by flash column chromatography (1:20 EtOAc-cyclohexane). Yield: 257 mg (82%). R_f = 0.31 (1:6 EtOAc-cyclohexane); $[\alpha]_D$ = +50.5 (*c* 1.0, DCM); IR: ν_{max} = 1747, 1716 $cm^{-1.}$

^{1}H NMR (300 MHz, $CDCl_3$): δ = 5.45 (t, 1 H, $J_{2,3}$ = $J_{3,4}$= 9.7 Hz, H-3), 4.96 (t, 1 H, $J_{4,5}$= 9.7 Hz, H-4), 4.92 (d, 1 H, $J_{1,2}$ = 3.7 Hz, H-1), 4.84 (dd, 1 H, H-2), 3.90 (ddd, 1 H, $J_{5,6a}$ = 3.0, $J_{5,6b}$ = 7.8 Hz, H-5), 3.42 (s, 3 H, OCH_3), 3.30 (m, 2 H, C*H*$_2$N), 2.79-2.55 (m,

4 H, C*H*$_2$S, H-6a. H-6b), 2.26 (m, 6 H, CH_2), 1.56 (m, 6 H, CH_2), 1.45 (s, 9 H, CMe_3), 1.26 (m, 66 H, CH_2), 0.89 (t, 9 H, $J_{H,H}$ = 6.5 Hz, H-6$_{Hex}$).

^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 172.9, 172.6, 172.5 (CO ester), 155.7 (CO carbamate), 96.6 (C-1), 79.4 (*C*Me_3), 71.4 (C-4), 70.9 (C-2), 69.7 (C-3), 69.6 (C-5), 55.4 (OMe), 39.6 (*C*H_2N), 34.2 (CH_2), 33.7 (*C*H_2S), 33.1 (C-6), 31.9, 29.4, 29.3, 29.2, 29.1 (CH_2), 28.4 (C*Me$_3$*), 24.9, 24.8, 22.6 (CH_2), 13.6 (CH_3).

ESIMS: *m/z* = 1006.8 $[M + Na]^+$. Anal. Calcd for $C_{32}H_{57}NO_{10}S$: C, 68.32; H, 10.75; N, 1.42; S, 3.26. Found: C, 68.20; H, 10.66; N, 1.33; S, 3.02.

Methyl 6-(2-aminoethylthio)-2,3,4-tri-*O*-myristoil-α-D-glucopyranoside (13). Treating of **12** (140 mg, 0.14 mmol) with 1:1 TFA-DCM (1 mL) as indicated in general methods yielded **13**. Yield: 120 mg (92%). $[\alpha]_D$ = + 62.2 (*c* 0.1, DCM); IR: ν_{max} = 3331 cm^{-1}.

^{1}H NMR (300 MHz, CD_3OD): δ = 5.41 (t, 1 H $J_{2,3}$ = $J_{3,4}$ = 9.5 Hz, H-3), 4.95 (t, 1 H, $J_{4,5}$ = 9.5 Hz, H-4), 4.87 (d, 1 H, $J_{1,2}$ = 3.6 Hz, H-1), 4.78 (dd, 1 H, H-2), 3.9 (ddd, 1 H, $J_{5,6a}$ = 3.0 Hz, $J_{5,6b}$ = 7.2 Hz, H-5), 3.37 (s, 1 H, OCH_3), 3.08 (m, 2 H, JH,H = 7.0 Hz, C*H*$_2$N), 2.87 (m, 2 H, C*H*$_2$S), 2.67 (dd, 1 H, $J_{6a,6b}$ = 14.5 Hz, H-6a), 2.56 (dd, 1 H, H-6b), 2.19 (m, 6 H, CH_2), 1.48 (m, 6 H, CH_2), 1.19 (m, 60 H, CH_2), 0.81 (m, 9 H, H-6$_{Hex}$).

^{13}C NMR (75.5 MHz, CD_3OD): δ = 173.1, 172.8, 172.7 (CO), 96.6 (C-1), 70.9 (C-4), 70.7 (C-2), 69.4 (C-3), 69.3 (C-5), 55.4 (OCH_3), 38.7 (*C*H_2N), 34.1 (CH_2), 32.7 (C-6), 30.4 (*C*H_2S), 31.8 (CH_2),29.6, 29.4, 29.3, 29.2, 29.8, 24.8, 24.7, 22.6 (CH_2), 14.0 (C-6$_{Hex}$).

ESIMS: *m/z* = 884.6 $[M]^+$. Anal. Calcd for $C_{27}H_{50}ClNO_8S$: C, 55.51; H, 8.63; N, 2.40; S, 5.49. Found: C, 63.21; H, 10.09; N, 3.86; S, 9.90

Methyl 6-(2-azidoethylthio)-2,3,4-tri-*O*-hexanoyl-α-D-glucopyranoside (14). To a solution of **11** (498 mg, 0.85 mmol), $NaHCO_3$ (289 mg, 3.48 mmol) and $CuSO_4 \cdot 5\ H_2O$ (8.5 mg, 0.034 mmol) in H_2O (3.5 mL), triflic azide (6 mL) and MeOH (23.6 mL) were added. Then, the blue mixture was stirred vigorously at rt for 24 h. The mixture was

concentrated at reduced pressure under 25 ºC and the residue was purified by column chromatography (1:4 EtOAc-cyclohexane). Yield: 435 mg (93%). $[\alpha]_D$ = + 90.7 (*c* 1.0, DCM); IR: ν_{max} = 2957, 2102, 1747 cm^{-1}.

^{1}H NMR (300 MHz, $CDCl_3$): δ = 5.45 (t, $J_{2,3} = J_{3,4}$ = 9.5 Hz, 1 H, H-3), 4.95 (t, 1 H, $J_{4,5}$ = 9.5 Hz, H-4), 4.90 (d, 1 H, $J_{1,2}$ = 3.7 Hz, H-1), 4.82 (dd, 1 H, H-2), 3.91 (ddd, 1 H, $J_{5,6b}$ = 7.6 Hz, $J_{5,6a}$ = 3.4 Hz, H-5), 3.43 (t, 2 H, $J_{H,H}$ = 7.0 Hz, CH_2N_3), 3.41 (s, 3 H, OMe), 2.74 (m, 2 H, CH_2S), 2.65 (m, 4 H, H-6a, H-6b), 2.23 (m, 6 H, H-2_{Hex}), 1.54 (m, 6 H, H-3_{Hex}), 1.25 (m, 12 H, H-4_{Hex}, H-5_{Hex}), 0.85 (t, 9 H, H-6_{Hex}).

^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 172.9-171.5 (CO), 96.6 (C-1), 71.4 (C-4), 70.9 (C-2), 69.9 (C-5), 69.5 (C-3), 55.4 (OMe), 51.2 (C-6), 34.1 (C-2_{Hex}), 33.6 (CH_2N), 31.2 (CH_2S), 31.2 (C-4_{Hex}), 24.5 (C-3_{Hex}), 22.2 (C-5_{Hex}), 13.8 (C-6_{Hex}).

ESIMS: *m/z* = 596.2 $[M + Na]^+$. Anal. Calcd for $C_{27}H_{47}N_3O_8S$: C, 56.52; H, 8.26; N, 7.32; S, 5.59. Found: C, 56.57; H, 8.29; N, 7.19; S, 5.42.

Methyl 2,3,4-tri-*O*-hexyl-6-*O*-trityl-α-D-glucopyranoside (15). To a solution of methyl 6-*O*-trityl-α-D-glucopyranoside **171** (700 mg, 1.6 mmol) in dry DMF (14 mL), NaH (573 mg, 14.43 mmol) was added and the mixture was stirred at 0 ºC for 10 min. 1-Bromohexane (2.02 mL, 14.43 mmol) was dropwise added, under Ar atmosphere, and the mixture was stirred overnight at rt. The reaction was quenched with MeOH (5 mL) for 10 min, the solvents were evaporated and DCM (10 mL) was added. The suspension was washed with H_2O (3 × 15 mL) and the organic layer was dried ($MgSO_4$), filtered, concentrated and purified by column chromatography (1:10 → 1:3 EtOAc-cyclohexane).Yield: 1.04 g (96%). R_f = 0.73 (1:3 EtOAc-cyclohexane); $[\alpha]_D$ = +44 (*c* 1.0, DCM); IR: ν_{max} = 1096, 1039 cm^{-1}.

^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.74-7.13 (m, 15 H, Ph), 4.83 (d, 1 H, $J_{1,2}$ = 3.6 Hz, H-1), 3.84-3.54 (m, 6 H,OCH_2), 3.51 (t, 1 H, $J_{2,3} = J_{3,4}$ = 9.5 Hz, H-3), 3.42 (s, 3 H, OCH_3), 3.37 (dd, 1 H, H-2), 3.37 (dd, 1 H, $J_{6a,6b}$ = 10.0 Hz $J_{5,6a}$ = 1.8 Hz, H-6a), 3.19 (t, 1

H, $J_{4,5}$ = 9.5 Hz, H-4), 3.14 (m, 1 H, H-5), 3.07 (dd, 1 H, $J_{5,6b}$ = 4.5 Hz, H-6b), 1.57 (m, 6 H, CH_2), 1.41-0.95 (m, 18 H, CH_2), 0.84 (m, 9 H, CH_3).

^{13}C NMR (75.5 MHz, $CDCl_3$): δ =144.3, 128.8, 127.8, 126.9 (Tr), 97.8 (C-1), 86.3 (Ph_3*C*), 81.8 (C-3), 80.8 (C-2), 78.3 (C-4), 73.8, 71.8, 70.4 (OCH_2), 73.2 (C-5), 62.5 (C-6), 54.8 (OCH_3), 31.8-22.6 (CH_2), 14.1 (CH_3).

ESIMS: *m/z* = 711.6 $[M + Na]^+$, 1400.8 $[2 M + Na]^+$. Anal. Calcd for $C_{44}H_{64}O_6$: C, 76.70; H, 9.36. Found: C, 76.87; H, 9.40.

Methyl 2,3,4-tri-*O*-hexyl-α-D-glucopyranoside (16). Trityl cleavage of **15** (130 mg, 0.19 mmol) was achieved with $BF_3{\cdot}Et_2O$ (170 μL) and MeOH (650 μL) in DCM (19 mL). The residue was purified by column chromatography (1:5 → 1:3 →1:1 EtOAc-cyclohexane). Yield: 390 mg (71%); R_f = 0.26 (1:3 EtOAc-cyclohexane); $[\alpha]_D$ = +81 (*c* 1.0, DCM).

^{1}H NMR (300 MHz, $CDCl_3$): δ = 4.72 (d, 1 H, $J_{1,2}$ = 3.5 Hz, H-1), 3.84-3.48 (m, 10 H, H-5, H-3, H-6b, OCH_2), 3.63 (s, 3 H, OCH_3), 3.20 (dd, 1 H, $J_{2,3}$ = 9.4 Hz, H-2), 3.37 (dd, 1 H, $J_{6a,6b}$ = 10.0 Hz, $J_{5,6a}$ = 1.8 Hz, H-6a), 3.19 (t, 1 H, $J_{3,4}$ = $J_{4,5}$ = 9.5 Hz, H-4), 1.55 (m, 6 H, CH_2), 1.41-1.18 (m, 18 H, CH_2), 0.86 (m, 9 H, CH_3).

^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 97.9 (C-1), 81.5 (C-3), 80.8 (C-2), 78.1 (C-4), 73.7, 71.8, 70.7 (OCH_2), 73.3 (C-5), 62.2 (C-6), 55.0 (OCH_3), 31.8-22.7 (CH_2), 13.9 (CH_3).

ESIMS: *m/z* = 469.6 $[M + Na]^+$, 915.7 $[2M + Na]^+$. Anal. Calcd for $C_{25}H_{50}O_6$: C, 67.22; H, 11.28. Found: C, 67.32; H, 11.12.

Methyl 2,3,4-tri-*O*-tetradecyl-6-*O*-trityl-α-D-glucopyranoside (17). To a solution of methyl 6-*O*-trityl-α-D-glucopyranoside **171** (700 mg, 1.6 mmol) in dry DMF (14 mL), NaH (364 mg, 14.4 mmol) was added and the mixture was stirred at rt for 10 min. 1-Bromotetradecane (2.5 mL, 14.4 mmol) was dropwise added, under Ar atmosphere, and the mixture was stirred overnight at 50 ºC. The reaction was quenched with MeOH (5 mL)

for 10 min, the solvents were evaporated and DCM (10 mL) was added. The suspension was washed with H_2O (3 × 15 mL) and the organic layer was dried ($MgSO_4$), filtered, concentrated and purified by column chromatography (1:50 → 1:40 EtOAc-cyclohexane).Yield: 1.36 g (83%); R_f = 0.53 (1:9 EtOAc-cyclohexane); $[\alpha]_D$ = +12 (*c* 1.0, DCM); IR: ν_{max} = 1098, 1041 cm^{-1}.

^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.52-7.07 (m, 15 H, Ph), 4.80 (d, 1 H, $J_{1,2}$ = 3.5 Hz, H-1), 3.79-3.52 (m, 6 H, OCH_2), 3.48 (t, 1 H, $J_{2,3}$ = $J_{3,4}$ = 9.5 Hz, H-3), 3.38 (s, 3 H, OCH_3), 3.34 (dd, 1 H, $J_{6a,6b}$ = 10.2 Hz, $J_{5,6a}$ = 1.8 Hz, H-6a), 3.29 (dd, 1 H, H-2), 3.24 (t, 1 H, $J_{4,5}$ = 9.5 Hz, H-4), 3.10 (m, 1 H, H-5), 3.04 (dd, 1 H, $J_{5,6b}$ = 4.5 Hz, H-6b), 1.54 (m, 6 H, CH_2), 1.37-1.03 (m, 66 H, CH_2), 0.82 (m, 9 H, CH_3).

^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 144.1, 128.8, 127.7, 126.9 (Ph), 97.9 (C-1), 86.2 (Ph_3C), 81.8 (C-3), 80.8 (C-2), 78.3 (C-4), 73.8, 71.8, 70.3 (OCH_2), 73.1 (C-5), 62.6 (C-6), 54.8 (OCH_3), 31.9-22.7 (CH_2), 14.1 (CH_3).

ESIMS: *m/z* = 1063.9 $[M + K]^+$, 1047.8 $[M + Na]^+$. Anal. Calcd for $C_{68}H_{112}O_6$: C, 79.63; H, 11.01. Found: C, 79.77; H, 11.10.

Methyl 2,3,4-tri-*O*-tetradecyl-α-D-glucopyranoside (18). Trityl cleavage of **17** (1.3 g, 1.26 mmol) was achieved was achieved with $BF_3{\cdot}Et_2O$ (173 μL) and MeOH (660 μL) in DCM (20 mL) and the residue was purified by column chromatography (1:8 → 1:6 → 1:4 EtOAc-cyclohexane). Yield: 723 mg (75%); R_f = 0.42 (1:4EtOAc-cyclohexane); $[\alpha]_D$ = +48 (*c* 1.0, DCM).

^{1}H NMR (300 MHz, $CDCl_3$): δ = 4.72 (d, 1 H, $J_{1,2}$ = 3.5 Hz, H-1), 3.86-3.49 (m, 10 H, H-5, H-6a, H-6b, H-3, OCH_2), 3.38 (s, 3 H, OCH_3), 3.20 (dd, 1 H, $J_{2,3}$ = 9.5 Hz, H-2), 2.19 (t, 1 H, $J_{3,4}$ = $J_{4,5}$ = 9.5 Hz, H-4), 1.55 (m, 6 H, CH_2), 1.36-1.18 (m, 18 H, CH_2), 0.86 (t, 9 H, $J_{H,H}$ = 6.7 Hz,CH_3).

^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 98.1 (C-1), 81.4 (C-3), 80.8 (C-2), 78.2 (C-4), 73.7, 71.8, 70.7 (OCH_2), 73.3 (C-5), 62.2 (C-6), 55.0 (OCH_3), 31.9-22.7 (CH_2), 14.1 (CH_3).

ESIMS: *m/z* = 805.7 $[M + Na]^+$. Anal. Calcd for $C_{49}H_{98}O_6$: C, 75.13; H, 12.61. Found: C, 74.98; H, 12.81.

Methyl 6-deoxy-2,3,4-tri-*O*-hexyl-6-iodo-α-D-glucopyranoside (19). To a solution of **16** (200 mg, 0.447 mmol) in toluene (9 mL), TPP (176 mg, 0.67 mmol) and imidazole (92 mg, 1.34 mmol) were added and the mixture was stirred at rt until complete dissolution. Iodine (177 mg, 0.63 mmol) was added in portions and the solution was vigorously stirred at 70 ºC for 5 h. A satd. $NaHCO_3$ solution (10 mL) was added and the mixture was further stirred 5 min. Additional iodine was then added until the aqueous solution got slightly brown, and then, a 10% $Na_2S_2O_8$ solution was added until complete decoloration of both organic and aqueous layer. The organic layer was then extracted, dried ($MgSO_4$), filtered, concentrated, and purified by column chromatography (1:7 EtOAc-cyclohexane). Yield: 227 mg (91%); R_f = 0.66 (1:4 EtOAc-cyclohexane); $[\alpha]_D$ = +75 (*c* 1.0, DCM).

^{1}H NMR (300 MHz, $CDCl_3$): δ = 4.75 (d, 1 H, $J_{1,2}$ = 3.6 Hz, H-1), 3.91-3.52 (m, 6 H, OCH_2), 3.48 (m, 1 H, H-6a), 3.42 (s, 3 H, OCH_3), 3.35 (m, 1 H, H-5), 3.34 (dd, 1 H, $J_{6a,6b}$ = 12.5 Hz, $J_{5,6b}$ = 5.6 Hz, H-6b), 3.24 (dd, 1 H, $J_{2,3}$= 9.6 Hz, H-2), 3.02 (t, 1 H, , H-4), 1.56 (m, 6 H, CH_2), 1.41-1.18 (m, 18 H, CH_2), 0.87 (m, 9 H, CH_3).

^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 98.1 (C-1), 81.8 (C-4), 81.1 (C-3), 80.6 (C-2), 73.7, 73.5, 71.8 (OCH_2), 69.4 (C-5), 55.4 (OCH_3), 31.8-22.6 (CH_2), 14.0 (CH_3), 8.0 (C-6).

ESIMS: *m/z* = 579.4 $[M + Na]^+$, 1135.4 $[2M + Na]^+$. Anal. Calcd for $C_{25}H_{49}IO_{52}$: C, 53.95; H, 8.87. Found: C, 54.08; H, 8.79.

Methyl 6-deoxy-6-iodo-2,3,4-tri-*O*-tetradecyl-α-D-glucopyranoside (20). To a solution of **18** (400 mg, 0.51 mmol) in toluene (18 mL), TPP (402 mg, 1.52 mmol) and imidazole (208 mg, 3.06 mmol) were added and the mixture was stirred at rt until complete dissolution. Iodine (406 mg, 1.42 mmol) was added in portions and the solution was vigorously stirred at 70 ºC for 5 h. A satd. $NaHCO_3$ solution (10 mL) was added and

the mixture was stirred for 5 min. Additional iodine was then added until the aqueous solution got slightly brown, and then, a 10% $Na_2S_2O_8$ solution was added until complete decoloration of both layers. The organic layer was then extracted, dried ($MgSO_4$), filtered, concentrated, and. purified by column chromatography (1:8 EtOAc-cyclohexane). Yield: 407 mg (89%); R_f = 0.77 (1:4 EtOAc-cyclohexane); $[\alpha]_D$ = +41 (*c* 1.0, DCM).

^{1}H NMR (300 MHz, $CDCl_3$): δ = 4.76 (d, 1 H, $J_{1,2}$ = 3.5 Hz, H-1), 3.90-3.46 (m, 8 H, H-3, H-6a, OCH_2), 3.43 (s, 3 H, OCH_3), 3.43 (m, 2 H, H-5, H-6b), 3.25 (dd, 1 H, $J_{2,3}$ = 9.6 Hz, H-2), 3.03 (t, 1 H, $J_{3,4}$ = 9.4 Hz, H-4), 1.56 (m, 6 H, CH_2), 1.41-1.17 (m, 66 H, CH_2), 0.87 (t, 9 H, $J_{H,H}$ = 7.0 Hz, CH_3).

^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 98.1 (C-1), 81.8 (C-4), 81.1 (C-3), 80.7 (C-2), 73.7, 73.5, 71.8 (OCH_2), 69.4 (C-5), 55.4 (OCH_3), 31.9 (CH_2), 30.6-29.3(CH_2), 28.6-22.7 (CH_2), 14.1 (CH_3), 8.0 (C-6).

ESIMS: *m/z* = 915.6 $[M + Na]^+$, 931.6 $[M + K]^+$. Anal. Calcd for $C_{49}H_{97}IO_5$: C, 65.89; H, 10.95. Found: C, 65.94; H, 10.80.

Methyl 6-azido-6-deoxy-2,3,4-tri-*O*-hexyl-α-D-glucopyranoside (21). To a solution of methyl 6-azido-6-deoxy-α-D-glucopyranoside **172** (400 mg, 1.82 mmol) in dry DMF (9 mL), NaH (656 mg, 16.42 mmol) was added, under Ar atmosphere, at 0 ºC. Then, 1-bromohexane (2.3 mL, 16.42 mmol) was added dropwise, and the reaction mixture was further stirred overnight at rt. The solvent was evaporated and the residue diluted in DCM (10 mL) and washed with H_2O (2 x 10 mL). The organic layer was dried ($MgSO_4$), concentrated and purified by column chromatography (1:30 EtOAc-cyclohexane). Yield: 500 mg (48%). R_f = 0.34 (1:18 EtOAc-cyclohexane); $[\alpha]_D$ = +90 (*c* 1.0, DCM); IR: ν_{max} = 2099, 1094 cm^{-1}.

^{1}H NMR (300 MHz, $CDCl_3$): δ = 4.74 (d, 1 H, $J_{1,2}$ = 3.5 Hz, H-1), 3.79, 3.64, 3.58 (m, 6 H, OCH_2), 3.67 (m, 1 H, H-5), 3.64-3.24 (m, 2 H, H-3, H-6a), 3.39 (s, 3 H, OCH_3), 3.35 (m, 1 H, H-6b), 3.23 (dd, 1 H, $J_{2,3}$ = 9.6 Hz, H-2), 3.12 (t, 1 H, $J_{3,4}$ = $J_{4,5}$ = 9.3 Hz, H-4), 1.55 (m, 6 H, CH_2), 1.27 (m, 18 H, CH_2), 0.86 (m, 9 H, CH_3).

^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 98.0 (C-1), 81.3 (C-3), 80.7 (C-2), 78.8 (C-4), 73.7, 73.6, 71.8 (OCH_2), 70.2 (C-5), 55.2 (OCH_3), 51.5 (C-6), 31.7-22.6 (CH_2), 14.3 (CH_3).

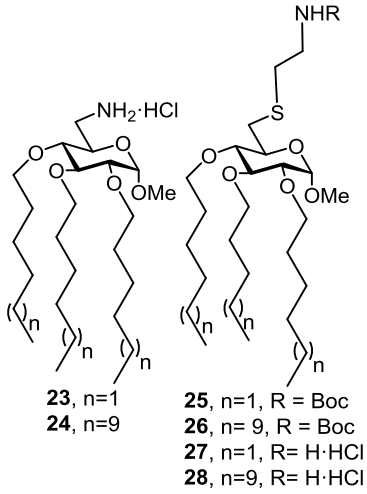
ESIMS: *m/z* = 494.6 $[M + Na]^+$, 965.8 $[2M + Na]^+$. Anal. Calcd for $C_{25}H_{49}N_3O_5$: C, 63.66; H, 10.47; N, 8.91. Found: C, 63.72; H, 10.97; N, 8.33.

Methyl 6-azido-6-deoxy-2,3,4-tri-*O*-tetradecyl-α-D-glucopyranoside (22). To a solution of methyl 6-azido-6-deoxy-α-D-glucopyranoside **172** (285 mg, 1.30 mmol) in dry DMF (4 mL), NaH (546 mg, 13.6 mmol) was added, under Ar atmosphere, at 0 ºC. Then, 1-bromotetradecane (4.1 mL, 13.6 mmol) was added dropwise, and the reaction mixture was further stirred overnight at 55 ºC. The solvent was evaporated and the residue diluted in DCM (10 mL) and washed with H_2O (2 x 10 mL). The organic layer was dried ($MgSO_4$), concentrated and purified by column chromatography (cyclohexane → 1:40 EtOAc-cyclohexane). Yield: 450 mg (52%). R_f = 0.25 (1:15 EtOAc-cyclohexane); $[\alpha]_D$ = +52 (*c* 1.0, DCM); IR: ν_{max} = 2100, 1096 cm^{-1}.

^{1}H NMR (300 MHz, $CDCl_3$): δ = 4.76 (d, 1 H, $J_{1,2}$ = 3.4 Hz, H-1), 3.79, 3.64, 3,58 (m, 6 H, OCH_2), 3.69 (m, 1 H, H-5), 3.66-3.44 (m, 2 H, H-3, H-6a), 3.39 (s, 3 H, OCH_3), 3.37 (dd, 1 H, $J_{6a,6b}$ = 13.3 Hz, $J_{5,6b}$ = 5.5 Hz, H-6b), 3.25 (dd, 1 H, $J_{2,3}$ = 9.4 Hz, H-2), 3.13 (t, 1 H, $J_{3,4}$ = $J_{4,5}$ = 9.4 Hz, H-4), 1.57 (m, 6 H, CH_2), 1.27 (m, 66 H, CH_2), 0.86 (m, 9 H, CH_3).

^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 98.0 (C-1), 81.3 (C-3), 80.7 (C-2), 78.8 (C-4), 73.7, 73.6, 71.8 (OCH_2), 70.2 (C-5), 55.2 (OCH_3), 51.5 (C-6), 31.9-22.7 (CH_2), 14.1 (CH_3).

ESIMS: *m/z* = 830.8 $[M + Na]^+$. Anal. Calcd for $C_{49}H_{97}N_3O_5$: C, 72.81; H, 12.10; N, 5.20. Found: C, 72.89; H, 11.87; N, 4.88.



Methyl 6-amino-6-deoxy-2,3,4-tri-*O*-hexyl-α-D-glucopyranoside hydrochloride (23). To a solution of **21** in degassed MeOH (12 mL), Pd/C (10%, 60 mg) was added and the mixture was stirred under H_2 atmosphere (1 bar) for 2 h at rt. The catalyst was filtered off, the solution concentrated, and the resulting residue purified by column chromatography (1:9 EtOAc-cyclohexane → 45:5:3 EtOAc-EtOH-H_2O) and freeze-dried from a solution of 0.1 N HCl. Yield: 123 mg (87%). $[\alpha]_D$ = +83 (*c* 1.0, DCM).

^{1}H NMR (300 MHz, CD_3OD): δ = 4.82 (d, 1 H, $J_{1,2}$ = 3.4 Hz, H-1), 3.81, 3.64, 3.56 (m, 6 H, OCH_2), 3.65 (m, 1 H, H-5), 3.53 (t, 1 H, $J_{2,3}$ = $J_{3,4}$ = 9.3 Hz, H-3), 3.41 (s, 3 H, OCH_3), 3.25 (dd, 1 H, H-2), 3.11 (dd, 1 H, $J_{6a,6b}$ = 13.1 Hz, $J_{5,6a}$ = 2.7 Hz, H-6a), 3.01 (t, 1 H, $J_{4,5}$ = 9.3 Hz, H-4), 2.84 (dd, 1 H, $J_{5,6b}$ = 8.5 Hz, H-6b), 1.56 (m, 6 H, CH_2), 1.33 (m, 18 H, CH_2), 0.91 (m, 9 H, CH_3).

^{13}C NMR (75.5 MHz, CD_3OD): δ = 99.1 (C-1), 82.6 (C-3), 81.8 (C-2), 81.1 (C-4), 74.5, 74.3, 72.2 (OCH_2), 70.7 (C-5), 55.8 (OCH_3), 42.9 (C-6), 33.0-24.2 (CH_2), 14.4 (CH_3).

ESIMS: *m/z* = 446.5 $[M + H]^+$, 891.7 $[2 M + H]^+$. Anal. Calcd for $C_{25}H_{52}ClNO_5$: C, 62.28; H, 10.87; N, 2.91. Found: C, 62.33; H, 10.69; N, 2.70.

Methyl 6-amino-6-deoxy-2,3,4-tri-*O*-tetradecyl-α-D-glucopyranoside hydrochloride (24). To a solution of **22** in THF (16.4 mL), TPP (104 mg, 0.39 mmol) was added and the mixture was stirred at rt for 15 min. Then NH_4OH (1.6 mL) was added and the solution was stirred overnight at 50 ºC. The mixture was concentrated and the resulting residue purified by column chromatography (EtOAc →1:9 DCM-MeOH).The residue was dissolved in 10:1 H_2O-HCl 0.1 N and freeze-dried to yield the product as hydrochloride. Yield: 128 mg (82%). $[\alpha]_D$ = +43 (*c* 1.0, DCM); IR: ν_{max} = 1092 cm^{-1}.

^{1}H NMR (300 MHz, $CDCl_3$): δ = 4.79 (d, 1 H, $J_{1,2}$ = 3.4 Hz, H-1), 3.78, 3.60, 3.55 (m, 7 H, OCH_2, H-3), 3.73 (m, 1 H, H-5), 3.46 (s, 3 H, OCH_3), 3.27 (m, 1 H, H-6a), 3.22 (dd, 1 H, $J_{2,3}$ = 9.5 Hz, H-2), 2.95 (m, 2 H, H-4, H-6b), 1.55 (m, 6 H, CH_2), 1.25 (m, 66 H, CH_2), 0.87 (t, 9 H, $^3J_{H,H}$ = 6.6 Hz, CH_3).

^{13}C NMR (75.5 MHz, $CDCl_3$): δ =98.0 (C-1), 81.0 (C-3), 80.4 (C-2), 79.9 (C-4), 77.2 (C-5), 73.7, 73.5, 71.8 (OCH_2), 67.1 (C-6), 55.9 (OCH_3), 31.9-22.7 (CH_2), 14.0 (CH_3).

ESIMS: *m/z* = 783.0 $[M + H]^+$. Anal. Calcd for $C_{49}H_{100}ClNO_5$: C, 71.88; H, 12.31; N, 1.71. Found: C, 71.64; H, 12.26; N, 1.49.

Methyl 6-(2-*tert*-butoxycarbonylaminoethylthio)-2,3,4-tri-*O*-hexyl-α-D-glucopyranoside (25). To a solution of **19** (430 mg, 0.76 mmol) in dry DMF (6.5 mL), Cs_2CO_3 (352 mg, 1.08 mmol) and *tert*-butyl *N*-(2-mercaptoethyl)carbamate (183 μL, 1.08 mmol) were added, under Ar atmosphere, and the reaction mixture was stirred at 60 ºC for 24 h. The reaction mixture was concentrated and the crude product was dissolved in DCM (10 mL) and washed with H_2O (2 x 15 mL). The organic phase was dried ($MgSO_4$), filtered, concentrated, and purified by column chromatography (1:8 → 1:6 EtOAc-cyclohexane)**.** Yield: 322 mg (70%). R_f = 0.35 (1:4 EtOAc-cyclohexane); $[\alpha]_D$ = +61 (*c* 1.0, DCM); IR: ν_{max} = 3350, cm^{-1}.

^{1}H NMR (300 MHz, $CDCl_3$): δ = 5.02 (bs, 1 H, NHBoc), 4.72 (d, 1 H, $J_{1,2}$ = 3.6 Hz, H-1), 3.86-3.45 (m, 8 H, H-5, H-3, OCH_2), 3.40 (s, 3 H, OCH_3), 3.31 (q, 2 H, $J_{H,H} = J_{H,NH}$

= 6.0 Hz, CH_2N), 3.22 (dd, 1 H, $J_{2,3}$ = 9.6 Hz, H-2), 3.07 (t, 1 H, $J_{3,4}$ = $J_{4,5}$ = 9.6 Hz, H-4), 2.89 (m, 1 H, $J_{6a,6b}$ = 13.7 Hz, $J_{5,6a}$ = 2.6 Hz, H-6a), 2.71 (t, 2 H, CH_2S), 2.65 (dd, 1 H, $J_{5,6b}$ = 7.5 Hz, H-6b), 1.55 (m, 6 H, CH_2), 1.42 (s, 9 H, CMe_3), 1.27 (m, 18 H, CH_2), 0.87 (m, 9 H, CH_3).

^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 155.7 (CO), 97.8 (C-1), 81.4 (C-3), 80.9 (C-2), 80.8 (C-4), 79.2 (*C*Me_3), 73.6, 73.4, 71.8 (CH_2), 70.9 (C-5), 55.0 (OCH_3), 39.7 (CH_2S), 33.6 (CH_2N), 33.5 (C-6), 31.8-30.0 (CH_2), 28.4 (CMe_3), 25.9 (CH_2), 22.6 (CH_2), 14.0 (CH_3).

ESIMS: *m/z* = 628.5 $[M + Na]^+$. Anal. Calcd for $C_{32}H_{63}NO_7S$: C, 63.43; H, 10.48; N, 2.31; S, 5.29. Found: C, 63.41; H, 10.37; N, 2.11; S, 4.96.

Methyl 6-(2-*tert*-butoxycarbonylaminoethylthio)-2,3,4-tri-*O*-tetradecyl-α-D-glucopyranoside (26). To a solution of **20** (336 mg, 0.37 mmol) in dry DMF (5.5 mL), Cs_2CO_3 (179 mg, 0.55 mmol) and *tert*-butyl *N*-(2-mercaptoethyl)carbamate (93 μL, 0.55 mmol) were added, under Ar atmosphere, and the reaction mixture was stirred at 60 ºC for 24 h. The reaction mixture was concentrated and the crude product was dissolved in DCM (10 mL) and washed with H_2O (2 x 15 mL). The organic phase was dried ($MgSO_4$), filtered, concentrated, and purified by column chromatography (1:8 → 1:6 EtOAc-cyclohexane). Yield: 253 mg (71%). R_f = 0.50 (1:2 EtOAc-cyclohexane); $[\alpha]_D$ = +40 (*c* 1.0, DCM).

^{1}H NMR (300 MHz, $CDCl_3$): δ = 5.02 (bs, 1 H, NHBoc), 4.72 (d, 1 H, $J_{1,2}$ = 3.7 Hz, H-1), 3.86-3.45 (m, 8 H, H-5, H-3, OCH_2), 3.40 (s, 3 H, OCH_3), 3.32 (m, 2 H, CH_2N), 3.22 (dd, 1 H, $J_{2,3}$= 9.5 Hz, H-2), 3.06 (t, 1 H, $J_{3,4}$ = $J_{4,5}$ = 9.5 Hz, H-4), 2.89 (m, 1 H, $J_{6a,6b}$ = 13.4 Hz, $J_{5,6a}$ = 2.4 Hz, H-6a), 2.71 (t, 2 H, $J_{H,H}$ = 6.0 Hz, CH_2S), 2.65 (dd, 1 H, $J_{5,6b}$ = 7.6 Hz, H-6b), 1.55 (m, 6 H, CH_2), 1.43 (s, 9 H, CMe_3), 1.26 (m, 66 H, CH_2), 0.87 (t, 9 H, $J_{H,H}$ = 6.5 Hz, CH_3).

^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 154.7 (CO), 96.8 (C-1), 81.4 (C-3), 80.9 (C-2), 80.8 (C-4), 79.3 (*C*Me_3), 73.6, 73.4, 71.8 (OCH_2), 70.9 (C-5), 55.0 (OCH_3), 39.7 (CH_2N), 33.6 (CH_2S), 33.5 (C-6), 31.9-28.5 (CH_2), 28.4 (C*Me*$_3$), 25.9, 22.6 (CH_2), 14.1 (CH_3).

ESIMS: *m/z* = 964.7 $[M + Na]^+$, 980.7 $[M + K]^+$. Anal. Calcd for $C_{42}H_{11}NO_7S$: C, 71.36; H, 11.87; N, 1.49; S, 3.40. Found: C, 71.44; H, 11.77; N, 1.38; S, 3.25.

Methyl 6-(2-aminoethylthio)-2,3,4-tri-*O*-hexyl-α-D-glucopyranoside hydrochloride (27). Compound **27** was obtained by treatment of **25** (237 mg, 0.39 mmol) with 1:1 TFA-DCM as indicated in general methods. Yield: 210 mg (99%). $[\alpha]_D$ = +90 (*c* 1.0, DCM).

^{1}H NMR (300 MHz, CD_3OD): δ = 4.80 (d, 1 H, $J_{1,2}$ = 3.5 Hz, H-1), 3.87-3.50 (m, 7 H, H-5, OCH_2), 3.45 (t, 1 H, $J_{2,3} = J_{3,4}$ = 9.5 Hz, H-3), 3.42 (s, 3 H, OCH_3), 3.26 (dd, 1 H, H-2), 3.16 (m, 3 H, CH_2N, H-4), 2.93 (m, 3 H, CH_2S, H-6a), 2.77 (m, 1 H, $J_{6a,6b}$ = 14.0 Hz, $J_{5,6b}$ = 6.9 Hz, H-6b), 1.56 (m, 6 H, CH_2), 1.32 (m, 18 H, CH_2), 0.90 (m, 9 H, CH_3).

^{13}C NMR (75.5 MHz, CD_3OD): δ = 99.0 (C-1), 82.8 (C-3), 81.9 (C-2), 81.8 (C-4), 74.6, 74.4, 72.6 (OCH_2), 72.3 (C-5), 55.6 (OCH_3), 40.0 (CH_2N), 34.1 (C-6), 33.02 (CH_2), 31.2 (CH_2S), 31.7 (CH_2), 25.9 (CH_2), 14.1 (CH_3).

ESIMS: *m/z* = 506.6 $[M + H]^+$. Anal. Calcd for $C_{32}H_{63}NO_7S$: C, 59.81; H, 10.41; N, 2.58; S, 5.91. Found: C, 59.64; H, 10.25; N, 2.39; S, 5.71.

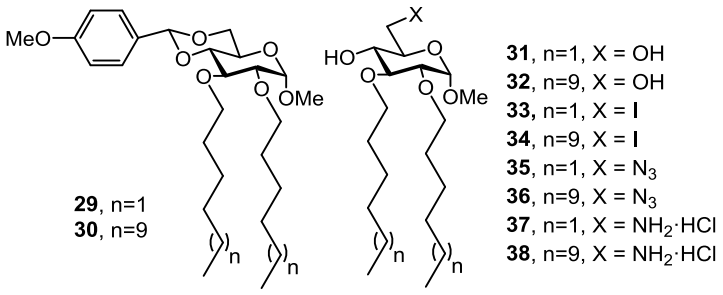
Methyl 6-(2-aminoethylthio)-2,3,4-tri-*O*-tetradecyl-α-D-glucopyranoside hydrochloride (28). Treatment of **26** (190 mg, 0.20 mmol) with 1:1 TFA-DCM (3 mL) was carried out as indicated in general methods. Yield: 170 mg (97%). $[\alpha]_D$ = +49 (*c* 1.0, DCM).

^{1}H NMR (300 MHz, $CDCl_3$): δ = 8.24 (bs, 3 H. NH_3), 4.72 (d, 1 H, $J_{1,2}$ = 3.4 Hz, H-1), 3.80-3.40 (m, 8 H, H-5, H-3, OCH_2), 3.34 (s, 3 H, OCH_3), 3.17 (dd, 1 H, $J_{2,3}$= 9.6 Hz, H-2), 3.15 (m, 2 H, CH_2N), 3.00 (t, 1 H, $J_{3,4} = J_{4,5}$ = 9.5 Hz, H-4), 2.91 (bt, 2 H, $J_{H,H}$ = 6.2 Hz,

CH_2S), 2.82 (m, 1 H, $J_{6a,6b}$ = 14.2 Hz, $J_{5,6a}$ = 2.3 Hz, H-6a), 2.65 (dd, 1 H, $J_{5,6b}$ = 7.1 Hz, H-6b), 1.49 (m, 6 H, CH_2), 1.20 (m, 66 H, CH_2), 0.81 (t, 9 H, $^3J_{H,H}$ = 6.6 Hz, CH_3).

^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 97.8 (C-1), 81.4 (C-3), 80.9 (C-2), 80.8 (C-4), 73.7, 73.7, 71.7 (OCH_2), 71.0 (C-5), 55.2 (OCH_3), 38.7 (CH_2N), 33.1 (C-6), 31.9 (CH_2), 30.4 (CH_2S), 30.5-22.6 (CH_2), 14.1 (CH_3).

ESIMS: *m/z* = 842.8 $[M]^+$. Anal. Calcd for $C_{51}H_{104}ClNO_5S$: C, 69.70; H, 11.93; N, 1.59; S, 3.65. Found: C, 69.82; H, 11.99; N, 1.38; S, 3.65.



Methyl 4,6-*O*-(4-methoxybenzylidene)-2,3-di-*O*-hexyl-α-D-glucopyranoside (29). To a solution of methyl 4,6-*O*-(4-methoxybenzylidene)-α-D-glucopyranoside **174** (0.80 g, 2.57 mmol) in DMF (8 mL), NaH (0.62 g, 15.42 mmol) was added. Then, 1-bromohexane (1.8 mL, 12.85 mmol) was added dropwise and the resulting mixture was stirred at 60 ºC overnight. After cooling to rt, the reaction was quenched with MeOH (2 mL) and the solution was stirred for 20 min. Solvents were then evaporated and the residue was diluted with EtOAc (50 mL) and citric acid (satd aq soln, 40 mL). The layers were separated and the organic phase was washed with H_2O (3 x 40 mL), dried ($MgSO_4$), evaporated, and purified by column chromatography (1:9 EtOAc-cyclohexane). Yield 0.71 g (57%). R_f = 0.44 (1:9 EtOAc-cyclohexane); $[\alpha]_D$ = +37.2 (*c* 1.0, DCM).

^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.39, 6.87 (2 d, 4 H, A_2X_2, aromatics), 5.49 (s, 1 H, PhC*H*), 4.78 (d, 1 H, $J_{1,2}$ = 3.8 Hz, H-1), 4.25 (dd, 1 H, $J_{6a,6b}$ = 9.6 Hz, $J_{5,6a}$ = 4.5 Hz, H-6a), 3.80 (s, 3 H, $PhOCH_3$), 3.76 (m, 1 H, H-5), 3.72 (t, 1 H, $J_{2,3}$ = $J_{3,4}$ = 9.2 Hz, H-3)

3.72-3.57 (m, 5 H, H-6b, OCH_2), 3.47 (t, 1 H, $J_{4,5}$ = 9.2 Hz, H-4), 3.42 (s, 3 H, OCH_3), 3.34 (dd, 1 H, H-2), 1.66-1.49 (m, 4 H, CH_2), 1.41-1.18 (m, 12 H, CH_2), 0.88, 0.84 (2 t, 6 H, $^3J_{H,H}$ = 6.5 Hz, CH_3).

^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 159.9-113.5 (Ph), 101.2 (Ph*C*H), 99.1 (C-1), 81.9 (C-4), 80.4 (C-2), 78.2 (C-3), 73.4, 72.2 (OCH_2), 69.0 (C-6), 62.4 (C-5), 55.6 (OCH_3), 31.7-22.6 (CH_2), 14.0 (CH_3).

ESIMS: *m/z* = 519.5 $[M + K]^+$, 503.6 $[M + Na]^+$. Anal. Calcd for $C_{27}H_{44}O_7$: C, 67.47; H, 9.23. Found: C, 67.54; H, 9.30.

Methyl 4,6-*O*-(4-methoxybenzylidene)-2,3-di-*O*-tetradecyl-α-D-glucopyranoside (30). To a solution of methyl 4,6-*O*-(4-methoxybenzylidene)-α-D-glucopyranoside **174** (0.80 g, 2.57 mmol) in DMF (8 mL), NaH (60% suspension in mineral oil, 0.62 g, 15.42 mmol) was carefully added in small portions. 1-Bromotetradecane (3.8 mL, 12.85 mmol) was added dropwise and the resulting mixture was stirred at 60 ºC overnight. After cooling to rt, the mixture was quenched with methanol (2 mL) then the solution was stirred for 20 min. Solvents were then evaporated and the residue was diluted with EtOAc (50 mL). Citric acid (satd aq soln, 40 mL) was added, the layers were separated, the organic layer was washed with H_2O (3 x 40 mL), dried (Na_2SO_4) and evaporated. Flash column chromatography on silica gel of the residue (1:9 EtOAc-cyclohexane) afforded **16.** Yield: 1.33 g (74%). R_f = 0.65 (1:9 EtOAc-cyclohexane); $[\alpha]_D$ = +23.3 (*c* 1.0 in $CHCl_3$).

^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.40, 6.87 (2 d, 4 H, A_2X_2, aromatics), 5.49 (s, 1 H, PhC*H*), 4.78 (d, 1 H, $J_{1,2}$ = 3.8 Hz, H-1), 4.25 (dd, 1 H, $J_{6a,6b}$ = 9.6, $J_{5,6a}$ = 4.6 Hz, H-6a), 3.80 (s, 3 H, OCH_3), 3.76 (m, 1 H, H-5), 3.75-3.59 (m, 6 H, H-3, H6b, OCH_2), 3.47 (t, 1 H, $J_{3,4}$ = $J_{4,5}$ = 9.3 Hz, H-4), 3.42 (s, 3 H, OCH_3), 3.34 (dd, 1 H, $J_{2,3}$ = 9.3, H-2), 1.70-1.49 (m, 4 H, CH_2), 1.22 (bs, 44 H, CH_2), 0.87 (t, 6 H, $J_{H,H}$ = 5.8 Hz, CH_3).

^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 159.1, 130.0, 127.3, 113.5 (Ph), 101.2 (Ph*C*H), 99.1 (C-1), 81.9 (C-4), 80.4 (C-2), 78.2 (C-3), 73.4, 72.3 (OCH_2), 69.0 (C-6), 62.4 (C-5), 55.2 (OCH_3), 31.9-22.7 (CH_2), 14.1 (CH_3).

ESIMS: *m/z* = 503.6 $[M + Na]^+$, 519.5 $[M + K]^+$. Los datos microanalíticos fueron idénticos a los descritos en la bibliografía.[15]

Methyl 2,3-di-*O*-hexyl-4-*O*-(*p*-methoxybenzyl)-α-D-glucopyranoside (31). To a solution of **29** (0.71 g, 1.48 mmol) in a mixture of Et_2O-DCM (2:1, 75 mL), under Ar atmosphere, 1 M $LiAlH_4$ in THF (7.4 mL, 7.40 mmol) and $AlCl_3$ (0.81 g, 6.06 mmol) in Et_2O (25 mL) were added dropwise, and the resulting mixture was refluxed for 4 h. After cooling to rt, EtOAc (250 mL) and H_2O (250 mL) were added. The organic layer was washed with brine (2 x 200 mL), dried ($MgSO_4$), evaporated and purified by column chromatography (1:2 EtOAc-cyclohexane). Yield: 0.60 g (83%). R_f = 0.26 (1:2 EtOAc-cyclohexane); $[\alpha]_D$ = +76.2 (*c* 1.0, DCM); IR: ν_{max} = 1076, 1035cm^{-1}.

^{1}H NMR (300 MHz, $CDCl_3$): δ =7.26, 6.87 (A_2X_2 system, 4 H, aromatics), 4.81 (d, 1 H, $^2J_{Ha,Hb}$ = 10.8 Hz, PhC*Ha*), 4.75 (d, 1 H, $J_{1,2}$ = 3.5 Hz, H-1), 4.57 (d, 1 H, PhC*Hb*), 3.86 (m, 1 H, OCH_2), 3.79 (s, 3 H, $PhOCH_3$), 3.77-3.64 (m, 3 H, H-6a, H-6b, OCH_2), 3.68 (m, 1 H, H-3), 3.67-3.53 (m, 3 H, OCH_2, H-5), 3.42 (t, 1 H, $J_{3,4}$ = $J_{4,5}$ = 9.5 Hz, H-4), 3.37 (s, 3 H, OCH_3), 3.26 (dd, 1 H, $J_{2,3}$ = 9.5 Hz, H-2), 1.75 (bs, 1 H, OH), 1.64-1.57 (m, 4 H, CH_2), 1.37-1.28 (m, 12 H, CH_2), 0.88 (t, 6 H, $J_{H,H}$ = 7.0 Hz, CH_3), 0.87 (t, 6 H, $J_{H,H}$ = 6.8 Hz, CH_3).

^{13}C NMR (75.5 MHz, $CDCl_3$): δ =159.3-113.8 (Ph), 98.0 (C-1), 81.6 (C-3), 80.8 (C-2), 77.1 (C-4), 74.5 ($PhCH_2$), 73.7, 71.7 (OCH_2), 70.6 (C-5), 62.0 (C-6), 55.2, 55.0 (OCH_3), 31.8-22.6 (CH_2), 14.0 (CH_3).

ESIMS: *m/z* = 505.6 $[M + Na]^+$, 521.5 $[M + K]^+$. Anal. Calcd for $C_{27}H_{46}O_7$: C, 67.19; H, 9.61. Found: C, 66.92; H, 9.67.

Methyl 2,3-di-*O*-tetradecyl-α-D-glucopyranoside (**32**). To a solution of **30** (0.696 g, 0.98 mmol) in a mixture of Et_2O-DCM (2:1, 15 mL), under Ar atmosphere, $AlCl_3$ (0.807 g, 6.06 mmol) in Et_2O (15 mL) were added dropwise and the resulting mixture was refluxed for 4 h. After cooling to rt, EtOAc (150 mL) and H_2O (150 mL) were added and the layers separated. The organic layer was washed with brine (3 x 100 mL), dried (Mg_2SO_4), evaporated and purified by column chromatography (1:1 EtOAc-cyclohexane). Yield: 502 mg (87%). R_f = 0.47 (1:1 EtOAc-cyclohexane); $[\alpha]_D$ = +24.7 (*c* 1 in DCM); IR: ν_{max} = 3362, 2953, 1468 cm^{-1}.

^{1}H NMR (300 MHz, $CDCl_3$): δ = 4.78 (d,1 H, $J_{1,2}$ = 3.5 Hz, H-1), 3.94-3.43 (m, 9 H, H-3, H,4 H-5, H-6a, H-6b, 2 OCH_2), 3.41 (s, 3 H, OCH_3), 3.26 (dd, 1 H, $J_{2,3}$ = 9.2 Hz, H-2), 1.69-1.48 (m, 4 H, CH_2), 1.25 (bs, 44 H, CH_2), 0.87 (t, 6 H, $J_{H,H}$ = 6.9 Hz, CH_3).

^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 98.1 (C-1), 81.0, 80.6 (C-2, C-3), 73.6 (C-4), 71.3, 70.7 (OCH_2), 70.5 (C-5), 62.5 (C-6), 55.2 (OCH_3), 31.9-22.6 (CH_2), 14.1 (CH_3).

ESIMS: *m/z* = 609.8 $[M + Na]^+$, 625.6 $[M + K]^+$. Anal. Calcd for $C_{35}H_{70}O_6$: C, 71.62; H, 12.02. Found: C, 71.38; H, 11.76.

Methyl 6-deoxy-2,3-di-*O*-hexyl-6-iodo-4-*O*-(*p*-methoxybenzyl)-α-D-glucopyranoside (33). To a solution of **31** (0.60 g, 1.23 mmol) in toluene (25 mL), TPP (0.49 g, 1.85 mmol) and imidazole (0.25 g, 3.70 mmol) were added. Iodine (0.49 g, 1.73 mmol) was added in portions and the resulting solution was stirred at 70 ºC for 5 h. After cooling at rt, saturated aqueous $NaHCO_3$ (25 mL) was added and the mixture was stirred for 5 min. Additional iodine was added and the mixture was stirred for 10 min. Then 10% aqueous $Na_2S_2O_3$ was added to remove the iodine excess. The organic layer was separated, washed with H_2O (3 x 25 mL), dried ($MgSO_4$), filtered, concentrated and purified by column chromatography (1:4 EtOAc-cyclohexane). Yield: 0.69 g (94%). R_f = 0.52 (1:4 EtOAc-cyclohexane); $[\alpha]_D$ = +84.3 (*c* 1.0, DCM).

^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.26, 6.88 (A_2X_2, 4 H, aromatics), 4.87 (d, 1 H, $^2J_{Ha,Hb}$ = 10.6 Hz, PhC*Ha*), 4.77 (d, 1 H, $J_{1,2}$ = 3.4 Hz, H-1), 4.62 (d, 1 H, PhC*Hb*), 3.87 (m, 1 H, CH_2), 3.80 (s, 3 H, $PhOCH_3$), 3.75-3.64 (m, 2 H, H-3, OCH_2), 3.63-3.50 (m, 2 H, OCH_2), 3.46 (dd, 1 H, $J_{6a,6b}$ = 10.4 Hz, $J_{5,6a}$ = 2.4 Hz, H-6a), 3.42 (s, 3 H, OCH_3), 3.37 (m, 1 H, H-5), 3.35-3.26 (m, 2 H, H-2, H-6b), 3.24 (t, 1 H, $J_{3,4}$ = $J_{4,5}$ = 9.0 Hz, H-4), 1.66-1.55 (m, 4 H, CH_2), 1.39-1.25 (m, 12 H, CH_2), 0.88 (t, 6 H, $J_{H,H}$ = 6.5 Hz, CH_3), 0.87 (t, 6 H, $J_{H,H}$ = 6.8 Hz, CH_3).

^{13}C NMR (75.5 MHz, $CDCl_3$): δ =159.4-113.9 (Ph), 98.0 (C-1), 81.2 (C-3), 81.1 (C-2), 80.8 (C-4), 74.9 ($PhCH_2$), 73.7, 71.7 (OCH_2), 69.2 (C-5), 55.4, 55.2 (OCH_3), 31.7-22.6 (CH_2), 14.0 (CH_3), 8.1 (C-6).

ESIMS: *m/z* = 631.3 $[M + K]^+$, 615.4 $[M + Na]^+$. Anal. Calcd for $C_{27}H_{45}IO_6$: C, 54.73; H, 7.65. Found: C, 54.88; H, 7.71.

Methyl 2,3-di-*O*-tetradecyl-6-deoxy-6-iodo-α-D-glucopyranoside (**34**). To a solution of **32** (0.495 g, 0.84 mmol) in toluene (17 mL), TPP (0.33 g, 1.26 mmol) and imidazole (0.17 g, 2.52 mmol) were added. Iodine (0.33 g, 1.17 mmol) was added in portions and the resulting solution was stirred at 70 ºC for 3 h. After conventional work-up, the organic layer was separated, washed with H_2O (3 x 20 mL), dried ($MgSO_4$), filtered, concentrated, and purified by column chromatography (1:9 EtOAc-cyclohexane). Yield: 532 mg (91%); R_f = 0.30 (1:9 EtOAc-cyclohexane); $[\alpha]_D$ = +44 (*c* 1, DCM); IR: ν_{max}= 1041, 722 cm^{-1}.

^{1}H NMR (300 MHz, $CDCl_3$): δ = 4.81 (d, 1 H, $J_{1,2}$ = 3.6 Hz, H-1), 3.91 (m, 1 H, OCH_2), 3.66-3.49 (m, 5 H, H-6a, OCH_2, H-3), 3.47 (s, 3 H, OCH_3), 3.45 (ddd, 1 H, $J_{4,5}$ = 9.2 Hz, $J_{5,6a}$ = 6.8 Hz, $J_{5,6b}$ = 2.2 Hz, H-5), 3.31 (m, 1 H, H-4) 3.29 (dd, 1 H, $J_{2,3}$ = 10.0 Hz, H-2), 3.26 (dd, 1 H, $J_{6a,6b}$ = 11.2 Hz, H-6b), 2.41 (d, 1 H, $J_{4,OH}$ = 2.3 Hz, OH-4), 1.61-1.54 (m, 4 H, CH_2), 1.25 (bs, 44 H, CH_2), 0.88 (t, 6 H, $J_{H,H}$ = 6.9 Hz, CH_3).

^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 98.2 (C-1), 80.7, 80.5 (C-2, C-3), 73.8 (C-4), 73.6, 71.2 (OCH_2), 69.7 (C-5), 55.5 (OCH_3), 31.9-22.7 (CH_2), 14.1 (CH_3), 7.2 (C-6).

ESIMS: *m/z* = 719.7 $[M + Na]^+$, 735.6 $[M + K]^+$. Anal. Calcd for $C_{35}H_{69}IO_5$: C, 60.33; H, 9.98. Found: C, 59.89; H, 9.72.

Methyl 6-azido-6-deoxy-2,3-di-*O*-hexyl-α-D-glucopyranoside (**35**). Compound **35** was obtained by treatment of **33** (0.242 g, 0.512 mmol) with NaN_3 (0.10 g, 1.537 mmol) in DMF (2.5 mL) and purification by column chromatography (1:5 EtOAc-cyclohexane). Yield: 185 mg, (93%). R_f = 0.53 (1:5 EtOAc-cyclohexane); $[\alpha]_D$ = +60 (*c* 0.9, DCM); IR: ν_{max} = 2097, 1051 cm^{-1}.

^{1}H NMR (300 MHz, $CDCl_3$): δ = 4.81 (d, 1 H, $J_{1,2}$ = 3.6 Hz, H-1), 3.92 (m, 1 H, OCH_2), 3.75 (ddd, 1 H, $J_{4,5}$ = 9.3 Hz, $J_{5,6a}$ = 6.0 Hz, $J_{5,6b}$ = 2.4 Hz, H-5), 3.64-3.42 (m, 6 H, H-6a, H-6b, OCH_2, H-3), 3.44 (s, 3 H, OCH_3), 3.39 (t, 1 H, $J_{3,4}$ = 9.3 Hz, H-4), 3.29 (dd, 1 H, $J_{2,3}$ = 9.3 Hz, H-2), 2.47 (bs, 1 H, OH-4), 1.63-1.52 (m, 4 H, CH_2), 1.38-1.24 (bs, 12 H, CH_2), 0.88, 0.87 (2 t, 6 H, $J_{H,H}$ = 7.2 Hz, CH_3).

^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 98.1 (C-1), 80.8, 80.6 (C-2, C-3), 73.6 (C-4), 71.2, 70.8 (OCH_2), 70.3 (C-5), 55.4 (OCH_3), 51.6 (C-6), 31.7, 31.6, 30.3, 30.0, 25.7, 25.6, 22.6 (CH_2), 14.0 (CH_3).

ESIMS: *m/z* = 360.5 $[M + H - N_2]^+$, 410.5 $[M + Na]^+$. Anal. Calcd for $C_{19}H_{37}N_3O_5$: C, 58.89; H, 9.62; N, 10.84. Found: C, 58.97; H, 9.71; N, 10.64.

Methyl 6-azido-6-deoxy-2,3-di-*O*-tetradecyl-α-D-glucopyranoside (**36**). Compound **36** was obtained by treatment of **34** (250 mg, 0.359 mmol) with NaN_3 (70 mg, 1.077 mmol) in DMF (2.5 mL) followed by column chromatography (1:5 EtOAc-cyclohexane). Yield: 198 mg (90%). R_f = 0.46 (1:5 EtOAc-cyclohexane); $[\alpha]_D$ = +39.9 (*c* 1.0 in DCM); IR: ν_{max} = 2099, 1039 cm^{-1}.

^{1}H NMR (300 MHz, $CDCl_3$): δ = 4.81 (d, 1 H, $J_{1,2}$ = 3.5 Hz, H-1), 3.93 (m, 1 H, OCH_2), 3.76 (ddd, 1 H, $J_{4,5}$ = 9.1 Hz, $J_{5,6a}$ = 6.1 Hz, $J_{5,6b}$ = 2.6 Hz, H-5), 3.64-3.49 (m, 5 H, H-6a, OCH_2, H-3), 349-3.35 (m, 2 H, H-6b, H-4), 3.45 (s, 3 H, OCH_3), 3.30 (dd, 1 H, $J_{2,3}$ = 9.3 Hz, H-2), 2.38 (d, 1 H, $J_{4,5}$ = 2.1 Hz, OH-4), 1.64-1.53 (m, 4 H, CH_2), 1.36-1.21 (bs, 48 H, CH_2), 0.88 (t, 6 H, $J_{H,H}$ = 7.1 Hz, CH_3).

^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 98.1 (C-1), 80.8, 80.6 (C-2, C-3), 73.6 (C-4), 71.2, 70.8 (OCH_2), 70.3 (C-5), 55.4 (OCH_3), 51.6 (C-6), 31.9, 30.4, 30.0, 29.7, 29.5, 29.4, 29.3, 26.1, 26.0, 22.7 (CH_2), 14.1 (CH_3).

ESIMS: *m/z* = 634.6 $[M + Na]^+$. Anal. Calcd for $C_{35}H_{69}N_3O_5$: calcd. C, 68.70; H, 11.37; N, 6.87. Found: C, 68.61; H, 11.22; N, 6.59.

Methyl 6-amino-6-deoxy-2,3-di-*O*-hexyl-α-D-glucopyranoside hydrochloride (**37**). Treatment of **35** (185 mg, 0.47 mmol) with TPP (250 mg, 0.955 mmol) in 10:1 THF-NH_4OH (22 mL) followed by purification by column chromatography (4:1 EtOAc-cyclohexane →EtOAc→ 45:5:3 EtOAc-EtOH-H_2O) and freeze-drying from a solution of 0.1 N HCl afforded **37**. Yield: 42 mg (54%); R_f = 0.57 (10:2:1MeCN-H_2O-NH_4OH); $[\alpha]_D$ = +74.1 (*c* 1.0, MeOH); IR: ν_{max} = 3397, 1096 cm^{-1}.

^{1}H NMR (300 MHz, CD_3OD): δ = 4.87 (d, 1 H, $J_{1,2}$ = 3.4 Hz, H-1), 3.77-3.53 (m, 5 H, OCH_2, H-5), 3.45 (s, 3 H, OCH_3), 3.43 (t, 1 H, $J_{2,3}$ = $J_{3,4}$ = 9.5 Hz H-3), 3.35 (dd, 1 H, $J_{6a,6b}$ = 13.1 Hz, $J_{5,6a}$ = 2.9 Hz, H-6a), 3.26 (dd, 1 H, H-2), 3.22 (dd, 1 H, $J_{4,5}$ = 9.0 Hz, H-4), 3.01 (dd, 1 H, $J_{5,6b}$ = 8.8 Hz, H-6a), 1.63-1.53 (m, 4 H, CH_2), 1.41-1.29 (bs, 12 H, CH_2), 0.91, 0.90 (2 t, 6 H, $J_{H,H}$ = 6.5 Hz, CH_3).

^{13}C NMR (75.5 MHz, CD_3OD): δ = 99.5 (C-1), 82.1, 81.3 (C-2, C-3), 74.7 (C-4), 73.2, 72.3 (OCH_2), 69.3 (C-5), 56.2 (OCH_3), 42.2 (C-6), 32.9-23.7 (CH_2), 14.4 (CH_3).

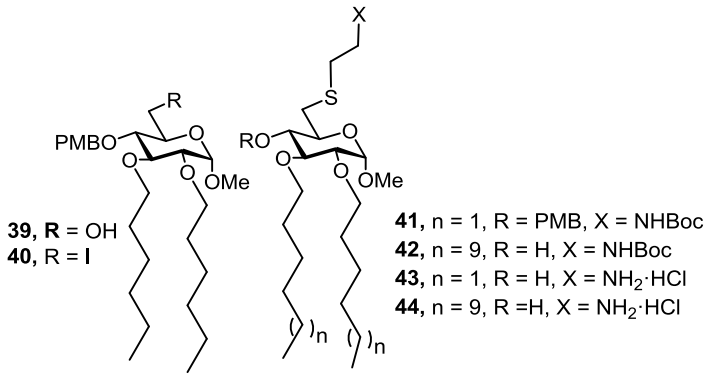
ESIMS: *m/z* = 362.4 $[M + H]^+$. Anal. Calcd for $C_{19}H_{40}NO_5$: C, 57.34; H, 10.13; N, 3.52. Found: C, 56.98; H, 10.02; N, 3.21.

Methyl 6-amino-6-deoxy-2,3-di-*O*-tetradecyl-α-D-glucopyranoside hydrochloride (**38**). Treatment of **36** (150 mg, 0.245 mmol) with TPP (128 mg, 0.39 mmol) in THF (20 mL) for 15 min at rt, followed by addition of NH_4OH (2.0 mL) and stirring overnight at 50 °C. The mixture was concentrated, the resulting residue was purified by column chromatography (4:1 EtOAc-cyclohexane → EtOAc → 45:5:3 AcOEt-EtOH-H_2O), dissolved in 10:1 H_2O-HCl 0.1 N and freeze-dried. Yield: 86 mg, (60%); R_f = 0.51 (10:2:1 MeCN-H_2O-NH_4OH).

^{1}H NMR (300 MHz, $CDCl_3$): δ = 4.74 (d, 1 H, $J_{1,2}$ = 3.7 Hz, H-1), 4.67 (bs, 2 H, NH_2), 3. 80 (m, 1 H, OCH_2), 3.86-3.49 (m, 4 H, OCH_2, H-5), 3.48 (t, 1 H, $J_{2,3}$ = $J_{3,4}$ = 9.3 Hz H-3), 3.39 (s, 3 H, OCH_3), 3.35 (dd, 1 H, $J_{5,6a}$ = 2.9 Hz, $J_{6a,6b}$ = 13.1 Hz, H-6a), 3.35 (dd, 1 H, $J_{4,5}$ = 9.3 Hz, H-4), 3.23 (dd, 1 H, H-2), 3.13-2.99 (m, 2 H, H-6ab), 1.92 (d, 1 H, OH-4), 1.61-1.51 (m, 4 H, CH_2), 1.33-1.20 (bs, 48 H, CH_2), 0.86 (t, 6 H, $J_{H,H}$ = 6.8 Hz, CH_3).

^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 98.2 (C-1), 80.9, 80.3 (C-2, C-3), 73.7 (C-4), 71.6, 71.5 (OCH_2), 69.5 (C-5), 56.2 (OCH_3), 42.0 (C-6), 31.9, 30.4, 30.1, 29.7, 29.6, 29.5, 29.3, 26.1, 26.0, 22.6 (CH_2), 14.0 (CH_3).

ESIMS: *m/z* = 586.5 $[M + H]^+$. Analitical data were according with those peviously described.[16]



Methyl 2,3-di-*O*-hexyl-4-*O*-(*p*-metoxybenzyl)-α-D-glucopyranoside (**39**). To a solution of **29** (0.71 g, 1.48 mmol) in a mixture of Et_2O-DCM (2:1, 75 mL), under Ar atmosphere, 1 M $LiAlH_4$ in THF (7.4 mL, 7.40 mmol) and $AlCl_3$ (0.81 g, 6.06 mmol) in

Et_2O (25 mL) were dropwise added, and the resulting mixture was refluxed for 4 h. After cooling to rt, EtOAc (250 mL) and H_2O (250 mL) were added. The organic layer was washed with brine (2 x 200 mL), dried ($MgSO_4$), evaporated.and purified by column chromatography (1:2 EtOAc-cyclohexane). Yield: 596 mg (83%). R_f = 0.26 (1:2 EtOAc-cyclohexane); $[\alpha]_D$ = +76 (*c* 1, DCM); IR: ν_{max}= 1076, 1035cm^{-1}.

^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.26, 6.87 (A_2X_2, 4 H, aromatics), 4.81 (d, 1 H, $^2J_{Ha,Hb}$ = 10.8 Hz, PhC*Ha*), 4.75 (d, 1 H, $J_{1,2}$ = 3.5 Hz, H-1), 4.57 (d, 1 H, PhC*Hb*), 3.86 (m, 1 H, OCH_2), 3.79 (s, 3 H, $PhOCH_3$), 3.77-3.64 (m, 3 H, H-6a, H-6b, OCH_2), 3.68 (m, 1 H, H-3), 3.67-3.53 (m, 3 H, OCH_2, H-5), 3.42 (t, 1 H, $J_{3,4}$ = $J_{4,5}$ = 9.5 Hz, H-4), 3.37 (s, 3 H, OCH_3), 3.26 (dd, 1 H, $J_{2,3}$ = 9.5 Hz, H-2), 1.75 (bs, 1 H, OH), 1.64-1.57 (m, 4 H, CH_2), 1.37-1.28 (m, 12 H, CH_2), 0.88 (t, 6 H, $J_{H,H}$ = 7.0 Hz, CH_3), 0.87 (t, 6 H, $J_{H,H}$ = 6.8 Hz, CH_3).

^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 159.3, 130.4, 129.7, 113.8 (Ph), 98.0 (C-1), 81.6 (C-3), 80.8 (C-2), 77.1 (C-4), 74.5 ($PhCH_2$), 73.7, 71.7 (OCH_2), 70.6 (C-5), 62.0 (C-6), 55.2, 55.0 (OCH_3), 31.8-22.6 (CH_2), 14.0 (CH_3).

ESIMS: *m/z* = 505.6 $[M + Na]^+$, 521.5 $[M + K]^+$. Anal. Calcd for $C_{27}H_{46}O_7$: C, 67.19; H, 9.61. Found: C, 66.92; H, 9.67.

Methyl 6-deoxy-2,3-di-*O*-hexyl-6-iodo-4-*O*-(*p*-metoxybenzyl)-α-D-glucopyranoside (**40**). To a solution of **39** (0.596 g, 1.235 mmol) in toluene (25 mL), TPP (0.486 g, 1.852 mmol) and imidazole (0.252 g, 3.705 mmol) were added. Iodine (0.490 g, 1.729 mmol) was added in portions and the resulting solution was stirred at 70 ºC for 5 h. After cooling at rt, saturated aqueous $NaHCO_3$ (25 mL) was added and the mixture was stirred for 5 min. Additional iodine was added up to turn brown the organic phase and the mixture was stirred for 10 min. Then, 10% aqueous $Na_2S_2O_3$ was added to remove the iodine excess. The organic layer was separated, washed with H_2O (3 × 25 mL), dried ($MgSO_4$), filtered, concentrated and purified by column chromatography

(EtOAc-cyclohexane 1:4). Yield: 687 mg (94%). R_f = 0.52 (1:4 EtOAc-cyclohexane); $[\alpha]_D$ = +84 (*c* 1, DCM).

^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.26, 6.88 (A_2X_2, 4 H, aromatics), 4.87 (d, 1 H, $J_{Ha,Hb}$ = 10.6 Hz, PhC*Ha*), 4.77 (d, 1 H, $J_{1,2}$ = 3.4 Hz, H-1), 4.62 (d, 1 H, PhC*Hb*), 3.87 (m, 1 H, CH_2), 3.80 (s, 3 H, $PhOCH_3$), 3.75-3.64 (m, 2 H, H-3, OCH_2), 3.63-3.50 (m, 2 H, OCH_2), 3.46 (dd, 1 H, $J_{6a,6b}$ = 10.4 Hz, $J_{5,6a}$ = 2.4 Hz, H-6a), 3.42 (s, 3 H, OCH_3), 3.37 (m, 1 H, H-5), 3.35-3.26 (m, 2 H, H-2, H-6b), 3.24 (t, 1 H, $J_{3,4}$ = $J_{4,5}$ = 9.0 Hz, H-4), 1.66-1.55 (m, 4 H, CH_2), 1.39-1.25 (m, 12 H, CH_2), 0.88 (t, 6 H, $J_{H,H}$ = 6.5 Hz, CH_3), 0.87 (t, 6 H, $J_{H,H}$ = 6.8 Hz, CH_3).

^{13}C NMR (75.5 MHz, $CDCl_3$): δ =159.4-113.9 (Ph), 98.0 (C-1), 81.2 (C-3), 81.1 (C-2), 80.8 (C-4), 74.9 ($PhCH_2$), 73.7, 71.7 (OCH_2), 69.2 (C-5), 55.4, 55.2 (OCH_3), 31.7-22.6 (CH_2), 14.0 (CH_3), 8.1 (C-6).

ESIMS: *m/z* = 631.3 $[M + K]^+$, 615.4 $[M + Na]^+$. Anal. Calcd for $C_{27}H_{45}IO_6$: C, 54.73; H, 7.65. Found: C, 54.88; H, 7.71.

Methyl 6-(2-*tert*-butoxycarbonylaminoethylthio)-2,3-di-*O*-hexyl-4-*O*-*p*-methoxybenzyl-α-D-glucopyranoside (41). To a suspension of **33** (0.69 g, 1.16 mmol) and Cs_2CO_3 (0.53 g, 1.62 mmol) in dry DMF (10 mL), *tert*-butyl (2-mercaptoethyl)carbamate (0.27 mL, 1.62 mmol) was added and the reaction mixture was stirred at 60 ºC, under Ar atmosphere, for 24 h. The mixture was concentrated, EtOAc (25 mL) and H_2O (25 mL) were added then the organic layer was separated, washed with H_2O (3 x 25 mL), dried ($MgSO_4$), filtered, concentrated and the residue was purified by column chromatography (1:6 → 1:4 EtOAc-cyclohexane). Yield: 0.74 g (99%). R_f = 0.17 (1:6 EtOAc-cyclohexane); $[\alpha]_D$ = +64.2 (*c* 1.0, DCM); IR: ν_{max}= 1714 cm^{-1}.

^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.24, 6.87 (A_2X_2, 4 H, aromatics), 4.98 (bs, 1 H, NH), 4.84 (d, 1 H, $J_{Ha,Hb}$ = 10.8 Hz, PhC*Ha*), 4.74 (d, 1 H, $J_{1,2}$ = 3.4 Hz, H-1), 4.54 (d, 1 H, PhC*Hb*), 3.85 (m, 1 H, OCH_2), 3.79 (s, 3 H, $PhOCH_3$), 3.74-3.52 (m, 3 H, OCH_2),

3.71 (m, 1 H, H-5), 3.64 (t, 1 H, $J_{2,3} = J_{3,4}$ = 9.3 Hz, H-3), 3.40 (s, 3 H, OCH_3), 3.31-3.25 (m, 4 H, H-2, H-4, CH_2N), 2.84 (dd, 1 H, $J_{6a,6b}$ = 13.9 Hz, $J_{5,6a}$ = 2.6 Hz, H-6a), 2.71 (t, 2 H, $J_{H,H}$ = 6.4 Hz, CH_2S), 2.57 (dd, 1 H, $J_{5,6b}$ = 7.5 Hz, H6b), 1.65-1.55 (m, 4 H, CH_2), 1.44 (s, 9 H, CMe_3), 1.36-1.24 (m, 12 H, CH_2), 0.88, 0.87 (2 t, 6 H, $J_{H,H}$ = 6.6 Hz, CH_3).

^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 159.3 (CO), 130.5-113.9 (Ph), 97.8 (C-1), 81.6 (C-3), 80.9 (C-2), 80.1 (C-4), 79.3 (*C*Me_3), 74.7 (CH_2Ph), 73.7, 71.7 (2 CH_2), 70.7 (C-5), 55.3, 55.0 (2 OCH_3), 39.7 (CH_2N), 33.6 (C-6), 33.5 (CH_2S), 31.7-29.6 (CH_2), 28.4 (C*Me*$_3$), 26.9-22.6 (CH_2), 14.0 (CH_3).

ESIMS: *m/z* = 664 $[M + Na]^+$, 680 $[M + K]^+$. Anal. Calcd for $C_{34}H_{59}NO_8S$: C, 63.62; H, 9.26; N, 2.18; S, 5.00. Found: C, 63.73; H, 9.21; N, 1.98; S, 4.86.

Methyl 6-(2-*tert*-butoxycarbonylaminoethylthio)-2,3-di-*O*-tetradecyl-α-D-glucopyranoside (**42**). To a suspension of **34** (0.53 g, 0.77 mmol) and Cs_2CO_3 (0.35 g, 1.07 mmol) in DMF (7 mL), *tert*-butyl (2-mercaptoethyl)carbamate (0.18 mL, 1.07 mmol) was added and the reaction mixture was stirred at 60 ºC, under Ar atmosphere for 24 h. The mixture was concentrated, EtOAc (20 mL) and H_2O (20 mL) were added and the layers separated. The organic layer was separated, washed with H_2O (3 × 20 mL), dried ($MgSO_4$), filtered, and concentrated. The residue was purified by column chromatography (EtOAc-cyclohexane 1:3). Yield: 542 mg (95%). R_f = 0.40 (1:3 EtOAc-cyclohexane); $[\alpha]_D$ = +50 (*c* 1, DCM); IR: ν_{max} = 3631, 1698, 1048 cm^{-1}.

^{1}H NMR (300 MHz, $CDCl_3$): δ = 4.99 (bs, 1 H, NH), 4.77 (d, 1 H, $J_{1,2}$ = 3.6 Hz, H-1), 3.90 (m, 1 H, OCH_2), 3.72 (ddd, 1 H, $J_{4,5}$ = 9.3 Hz, $J_{5,6a}$ = 7.5 Hz, $J_{5,6b}$ = 2.6 Hz, H-5), 3.64-3.46 (m, 3 H, OCH_2), 3.48 (t, 1 H, $J_{2,3} = J_{3,4}$ = 9.3 Hz, H-3), 3.43 (s, 3 H, OCH_3), 3.36 (t, 1 H, H-4), 3.31 (m, 2 H, CH_2N), 3.27 (dd, 1 H, H-2), 2.98 (dd, 1 H, $J_{6a,6b}$ = 14.1 Hz, H-6a), 2.71 (m, 3 H, H6b, CH_2S), 2.63 (bs, 1 H, OH-4), 1.65-1.50 (m, 4 H, CH_2), 1.43 (s, 9 H, CMe_3), 1.24 (bs, 44 H, CH_2), 0.87 (t, 6 H, $J_{H,H}$ = 7.0 Hz, CH_3).

^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 156 (CO), 97.9 (C-1), 80.9 (C-3), 80.6 (C-2), 79.4 (CMe_3), 73.5 (OCH_2), 72.4 (C-4), 71.2 (OCH_2), 71.0 (C-5), 55.2 (OCH_3), 39.6 (CH_2N), 33.5 (C-6), 33.4 (CH_2S), 31.8-29.3 (CH_2), 28.4 (CMe_3), 26.1-22.6 (CH_2), 14.0 (CH_3).

ESIMS: *m/z* = 768.8 $[M + Na]^+$, 784.7 $[M + K]^+$. Anal. Calcd for $C_{42}H_{83}NO_7S$: C, 67.60; H, 11.21; N, 1.88; S, 4.30. Found: C, 67.45; H, 10.90; N, 1.62; S, 4.24.

Methyl 6-(aminoethylthio)-2,3-di-*O*-hexyl-α-D-glucopyranoside hydrochloride (43). Treatment of **41** (0.35 g, 0.55 mmol) with 1:1 TFA-DCM (2 mL) and freeze-drying from 10:1 H_2O-0.1 N HCl solution afforded **43**. Yield: 0.20 g (quantitative). R_f = 0.45 (45:5:3 EtOAc-EtOH-H_2O); $[\alpha]_D$ = +74.4 (*c* 1.0, MeOH); IR: ν_{max} = 3404, 1109 cm^{-1}.

^{1}H NMR (300 MHz, CD_3OD): δ = 4.80 (d, 1 H, $J_{1,2}$ = 3.9 Hz, H-1), 3.74 (t, 1 H, $J_{H,H}$ = 6.8 Hz, OCH_2), 3.67-3.51 (m, 4 H, H-5, OCH_2), 3.42 (s, 3 H, OCH_3), 3.40 (t, 1 H, $J_{2,3}$ = $J_{3,4}$ = 9.3 Hz, H-3), 3.29 (t, 1 H, $J_{4,5}$ = 9.3 Hz, H-4), 3.23 (dd, 1 H, H-2), 3.16 (t, 2 H, $J_{H,H}$ = 6.8 Hz, CH_2N), 2.99 (dd, 1 H, $J_{6a,6b}$ = 14.4 Hz, $J_{5,6a}$ = 2.3 Hz, H-6a), 2.90 (m, 2 H, CH_2S), 2.72 (dd, 1H, $J_{5,6b}$ = 8.0 Hz, H6b), 1.60-1.52 (m, 4 H, CH_2), 1.42-1.31 (bs, 12 H, CH_2), 0.90, 0.89 (2 t, 6 H, $J_{H,H}$ = 6.6 Hz, CH_3).

^{13}C NMR (75.5 MHz, CD_3OD): δ = 99.1 (C-1), 82.6 (C-3), 81.6 (C-2), 74.6 (OCH_2), 74.0 (C-4), 73.4 (C-5), 72.2 (OCH_2), 55.5 (OCH_3), 40.0 (CH_2N), 34.2 (C-6), 33.0-31.1 (CH_2), 31.1 (CH_2S), 26.9, 26.8, 23.7 (CH_2), 14.4 (CH_3).

ESIMS: *m/z* = 422.5 $[M - Cl]^+$. Anal. Calcd for $C_{21}H_{43}NO_5S{\cdot}HCl$: C, 55.06; H, 9.68; N, 3.06; S, 7.00. Found: C, 54.87; H, 9.45; N, 2.79; S, 6.78.

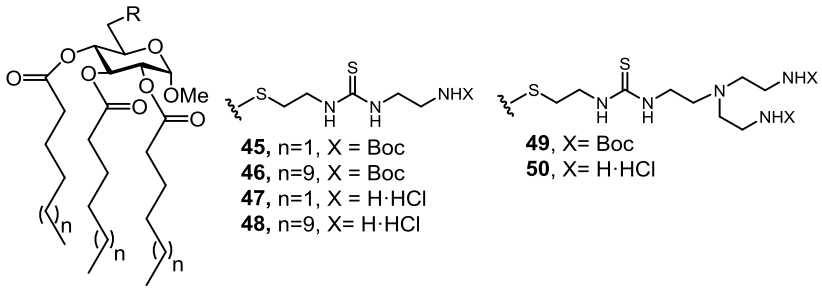
Methyl 6-(aminoethylthio)-2,3-di-*O*-tetradecyl-α-D-glucopyranoside hydrochloride (**44**). Treatment of **43** (246 mg, 0.33 mmol) with 1:1 DCM-TFA (4 mL) as indicated in general methods followed by column chromatography of the residue (4:1 EtOAc-cyclohexane → EtOAc →45:5:3 EtOAc-EtOH-H_2O) yielded quantitatively

44.Yield: 225 mg. R_f = 0.25 (45:5:3 EtOAc-EtOH-H_2O); $[\alpha]_D$ = +41 (*c* 0.9, 9:1 DCM-MeOH); IR: ν_{max} = 3406, 722 cm^{-1}.

^{1}H NMR (300 MHz, $CDCl_3$): δ = 8.11 (bs, 2 H, NH_2HCl), 4.78 (d, 1 H, $J_{1,2}$ = 3.2 Hz, H-1), 3.87 (m, 1 H, OCH_2), 3.74 (ddd, 1 H, $J_{4,5}$ = 9.4 Hz, $J_{5,6a}$ = 2.7 Hz, $J_{5,6b}$ = 6.7 Hz, H-5), 3.66-3.54 (m, 3 H, OCH_2), 3.49 (t, 1 H, $J_{2,3}$ = $J_{3,4}$ = 8.9 Hz, H-3), 3.42 (s, 3 H, OCH_3), 3.38 (t, 1 H, H-4), 3.27 (dd, 1 H, H-2), 3.18 (t, 2 H, $J_{H,H}$ = 6.2 Hz, CH_2N), 2.97 (dd, 1 H, $J_{6a,6b}$ = 14.2 Hz, H-6a), 2.90 (m, 2 H, CH_2S), 2.74 (dd, 1H, H6b), 1.60-1.54 (m, 4 H, CH_2), 1.25 (bs, 44 H, CH_2), 0.87 (t, 6 H, $J_{H,H}$ = 6.9 Hz, CH_3).

^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 97.9 (C-1), 80.9 (C-3), 80.5 (C-2), 73.7 (OCH_2), 72.1 (C-4), 71.3 (OCH_2), 70.7 (C-5), 55.2 (OCH_3), 38.7 (CH_2N), 33.2 (C-6), 31.9 (CH_2), 30.3 (CH_2S), 30.3, 30.1, 29.7, 29.6, 29.5, 29.4 26.0, 22.7 (CH_2), 14.1 (CH_3).

ESIMS: *m/z* = 646.7 $[M - HCl]^+$. Anal. Calcd for $C_{37}H_{76}ClNO_5S \cdot 2H_2O$: C, 61.85; H, 11.22; N, 1.95; S, 4.46. Found: C, 61.79; H, 11.03; N, 2.01; S, 4.43.



Methyl 6-(2-(*N´*-(2-(*N*-*tert*-butoxycarbonylamino)ethyl)thioureido)-ethyltio)-2,3,4-tri-*O*-hexanoyl-α-D-glucopyranoside (45). To a solution of **34** (160 mg, 0.27 mmol) and Et_3N (58 μL, 0.42 mmol) in DCM (12 mL), *tert*-butyl *N*-(2-isothiocyanoethyl)carbamate **168** (85 mg, 0.42 mmol) was added and the reaction mixture was stirred, under Ar atmosphere, at rt for 36 h. The reaction mixture was washed with aqueous diluted HCl (2 x 20 mL), dried ($MgSO_4$), filtered and concentrated. The residue was purified by column chromatography (1:2 → 1:1 EtOAc-cyclohexane). Yield: 84 mg

(42%). R_f = 0.67 (2:1 EtOAc-cyclohexane); $[\alpha]_D$ = + 69.4 (*c* 1, DCM); UV (DCM): λ_{max} = 251 nm (ε_{mM} 31.2); IR: ν_{max} = 2958, 1747, 1685 cm^{-1}.

^{1}H NMR (300 MHz, $CDCl_3$): δ = 6.80, 6.54 (bs, 2 H, N*H*CS), 5.45 (t, 1 H, $J_{3,4}$ = $J_{2,3}$ = 10.0 Hz, H-3), 5.02 (bs, 1 H, N*H*Boc), 4.99 (t, 1 H, $J_{4,5}$ = 10.0 Hz, H-4), 4.91 (d, 1 H, $J_{1,2}$ = 3.8 Hz, H-1), 4.83 (dd, 1 H, H-2), 3.93 (ddd, 1 H, $J_{5,6a}$ = 7.5 Hz, $J_{5,6b}$ = 2.8 Hz, H-5), 3.63 (bs, 2 H, SCH_2CH_2NHCS), 3.56 (bs, 2 H, CH_2CH_2NHBoc), 3.41 (s, 3 H, OCH_3), 3.30 (q, 2 H, $J_{H,H}$ = 6.0 Hz, CH_2NHBoc), 2.82 (m, 2 H, CH_2CH_2S), 2.72 (dd, 1 H, $J_{6a,6b}$ = 14.3 Hz, H-6a), 2.61 (dd, 1 H, H-6b), 2.33-2.16 (m, 6 H, H-2_{Hex}), 1.53 (m, 6 H, H-3_{Hex}), 1.42 (s, 9 H, CMe_3), 1.26 (m, 12 H, H-4_{Hex}, H-5_{Hex}), 0.85 (m, 9 H, H-6_{Hex}).

^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 172.1-171.2 (CO ester), 96.9 (C-1), 71.3 (C-4), 70.9 (C-2), 69.6 (C-5), 55.6 (OMe), 42.16 (SCH_2CH_2NHCS), 39.7 (CH_2NHBoc), 34.0 (C-2_{Hex}), 32.9 (C-6), 32.3 (SCH_2CH_2NHCS), 31.3 (C-4_{Hex}), 28.3 (CMe_3), 24.5 (C-3_{Hex}), 22.3 (C-5_{Hex}), 13.8 (C-6_{Hex}).

ESIMS: *m/z* = 772.4 $[M + Na]^+$. Anal. Calcd for $C_{35}H_{63}N_3O_{10}S_2$: C, 56.05; H, 8.47; N, 5.60; S, 8.55. Found: C, 56.59; H, 8.73; N, 5.31; S, 8.02.

Methyl 6-(2-(*N´*-(2-(*N*-*tert*-butoxycarbonylamino)ethyl)thioureido)ethyltio)-2,3,4-tri-*O*-myristoil-α-D-glucopyranoside (46). To a solution of **13** (94 mg, 0.102 mmol) and Et_3N (22 μL, 0.15 mmol) in DCM (8 mL), **168** (31 mg, 0.15 mmol) was added and the reaction mixture was stirred, under Ar atmosphere, at rt for 36 h. The reaction mixture was washed with aqueous diluted HCl (2 x 20 mL), dried ($MgSO_4$), filtered, concentrated andpurified by column chromatography (1:4 → 1:3 EtOAc-cyclohexane). Yield: 80 mg (72%). R_f = 0.63 (1:1 EtOAc-cyclohexane); $[\alpha]_D$ = + 37.9 (*c* 1.1, DCM); UV (DCM): λ_{max} = 250 nm (ε_{mM} 8.49); IR: ν_{max} = 3313, 1745, 1686 cm^{-1}.

^{1}H NMR (300 MHz, 10:1 $CDCl_3$-CD_3OD): δ = 5.61 (bs, 2 H, N*H*CS), 5.36 (t, 1 H, $J_{2,3}$ = $J_{3,4}$ = 9.7 Hz, H-3), 4.89 (t, 1 H, $J_{4,5}$ = 9.7 Hz, H-4), 4.81 (d, 1 H, $J_{1,2}$ = 3.7 Hz, H-1), 4.74 (dd, 1 H, H-2), 3.84 (ddd, 1 H, $J_{5,6b}$ = 7.7 Hz, $J_{5,6a}$ = 2.7 Hz, H-5), 3.56 (bs, 2 H,

SCH_2CH_2NHCS), 3.54 (bs, 2 H, CH_2CH_2NHBoc), 3.32 (s, 3 H, OCH_3), 3.15 (q, 2 H, $^3J_{H,H}$ = $^3J_{H,NH}$ = 6.1 Hz, CH_2NHBoc), 2.71 (m, 2 H, CH_2CH_2S), 2.64 (dd, 1 H, $J_{6a,6b}$ = 14.4 Hz, H-6a), 2.63 (dd, 1 H, H-6b), 2.26-2.05 (m, 6 H, CH_2), 1.44 (m, 6 H, CH_2), 1.33 (s, 9 H, CMe_3), 1.15 (m, 12 H, CH_2), 0.76 (t, 9 H, $J_{H,H}$ = 6.5 Hz, CH_3).

^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 184.1 (CS), 173.1, 172.9, 172.8 (CO ester), 157.5 (CO carbamate), 96.5 (C-1), 79.8 (CMe_3), 71.2 (C-4), 70.8 (C-2), 69.6 (C-5), 69.4 (C-3), 55.3 (OCH_3), 46.5 (CH_2CH_2NHBoc), 43.2 (SCH_2CH_2NHCS), 39.7 (CH_2NHBoc), 34.0 (CH_2), 32.9 (C-6), 32.2 (SCH_2), 31.8, 29.6, 29.5, 29.4, 29.3, 29.2, 29.1, 29.0, 28.9, 28.8 (CH_2), 28.2 (CMe_3), 24.8 (CH_2), 22.5 (CH_2), 13.8 (CH_3).

ESIMS: *m/z* = 1108.7 $[M + Na]^+$, 1124.6 $[M + K]^+$. Anal. Calcd for $C_{35}H_{63}N_3O_{10}S_2$: C, 65.21; H, 10.30; N, 3.87; S, 5.90. Found: C, 65.33; H, 10.41; N, 3.75; S, 5.66.

Methyl 6-(2-(*N´*-(2-aminoethyl)thioureido)ethyltio)-2,3,4-tri-*O*-hexanoyl-α-D-glucopyranoside hydrochloride (47). Compound **47** was obtained by treatment of **45** (121 mg, 0.16 mmol) with 1:1 TFA-DCM (1.6 mL) as indicated in general methods. Yield: 97.4 mg (86%). $[\alpha]_D$ = + 68.9 (*c* 1, MeOH); UV (MeOH): λ_{max} = 244 nm (ε_{mM} 89.6); IR: ν_{max} = 2955, 2929, 1747, 1551 cm^{-1}.

^{1}H NMR (500 MHz, CD_3OD): δ = 5.41 (t, 1 H, $J_{3,4}$ = $J_{2,3}$ = 9.5 Hz, H-3), 5.01 (t, 1 H, $J_{4,5}$ = 9.5 Hz, H-4), 4.92 (d, 1 H, $J_{1,2}$ = 3.8 Hz, H-1), 4.86 (dd, 1 H, H-2), 3.95 (ddd, 1 H, $J_{5,6b}$ = 7.1 Hz, $J_{5,6}$ = 2.8 Hz, H-5), 3.84 (t, 2 H, $J_{H,H}$ = 6.5 Hz, $NH_2CH_2CH_2NHCS$), 3.68 (bs, 2 H, SCH_2CH_2NHCS), 3.45 (s, 3 H, OCH_3), 3.16 (t, 2 H, $J_{H,H}$ = 6.5 Hz, CH_2NH_2), 2.81 (m, 3 H, CH_2CH_2S, H-6a), 2.68 (dd, 1 H, $J_{6a,6b}$ = 14.3 Hz, H-6b), 2.4-2.2 (m, 6 H, H-2_{Hex}), 1.56 (m, 6 H, H-3_{Hex}), 1.30 (m, 12 H, H-4_{Hex}, H-5_{Hex}), 0.90 (m, 9 H, H-6_{Hex}).

^{13}C NMR (125 MHz, $CDCl_3$): δ = 172.8-172.2 (CO), 97.2 (C-1), 96.5 (C-1), 71.2 (C-4), 70.8 (C-2), 69.2 (C-3), 69.5 (C-5), 54.4 (OMe), 43.6 (SCH_2CH_2NHCS), 40.9 ($NH_2CH_2CH_2NHCS$), 39.5 ($CH_2CH_2NH_2$), 32.7 (C-6), 31.0 (SCH_2CH_2), 33.5 (C-2_{Hex}), 30.8 (C-4_{Hex}), 24.4 (C-3_{Hex}), 22.1 (C-5_{Hex}), 13.2 (C-6_{Hex}).

ESIMS: *m/z* = 650.4 $[M + H]^+$ Anal. Calcd for $C_{30}H_{55}N_3O_8S_2$: C, 52.50; H, 8.22; N, 6.12; S, 9.34. Found: C, 56.41; H, 8.11; N, 5.98; S, 9.13.

Methyl 6-(2-(*N´*-(2-aminoethyl)thioureido)ethyltio)-2,3,4-tri-*O*-myristoil-α-D-glucopyranoside hydrochloride (48). Compound **48** was obtained by treatment of **46** (75 mg, 0.07 mmol) with 1:1 TFA-DCM (1 mL) as indicated in general methods. Yield: 60 mg (84%). $[\alpha]_D$ = + 41.3 (*c* 1, DCM); UV (DCM): λ_{max} = 249 nm (ε_{mM} 8.00); IR: ν_{max} = 3248, 1746 cm^{-1}.

^{1}H NMR (500 MHz, $CDCl_3$): δ = 8.07 (bs, 2 H, NH_2), 7.62 (bs, 1 H, NHCS), 7.16 (bs, 1 H, NHCS), 5.46 (t, 1 H, $J_{2,3} = J_{3,4} =$ 9.5 Hz, H-3), 5.02 (t, 1 H, $J_{4,5}$ = 9.5 Hz, H-4), 4.93 (d, 1 H, $J_{1,2}$ = 3.5 Hz, H-1), 4.89 (dd, 1 H, H-2), 3.96 (m, 3 H, H-5, $NH_2CH_2CH_2NHCS$), 3.70 (bs, 2 H, SCH_2CH_2NHCS), 3.43 (s, 3 H, OCH_3), 3.35 (bm, 2 H, CH_2NH_2), 2.85 (bt, 2 H, $J_{H,H}$ = 6.2 Hz, CH_2S), 2.67 (m, 2 H, H-6a, H-6b), 2.41-2.10 (m, 6 H, CH_2), 1.54 (m, 6 H, CH_2), 1.26 (m, 60 H, CH_2), 0.87 (m, 9 H, CH_2).

^{13}C NMR (125 MHz, $CDCl_3$): δ = 183.8 (CS), 173.7, 173.4, 173.3 (CO), 96.9 (C-1), 71.8 (C-4), 71.4 (C-2), 70.2 (C-3), 70.0 (C-5), 55.7 (OCH_3), 44.1 (SCH_2CH_2NHCS), 41.5 ($NH_2CH_2CH_2NHCS$), 40.1 ($CH_2CH_2NH_2$), 34.5 (CH_2), 33.4 (C-6), 32.5 (SCH_2), 32.3-23.0 (CH_2), 14.3 (CH_3).

ESIMS: *m/z* = 986.7 $[M]^+$. Anal. Calcd for $C_{54}H_{104}ClN_3O_8S_2$: C, 63.40; H, 10.25; N, 4.11; S, 6.27. Found: C, 63.13; H, 9.98; N, 3.85; S, 5.94.

Methyl 6-(2-(*N*'-(2-(*N*,*N*-di-(2-(*N*-*tert*-butoxycarbonylamino)ethyl)amino)-ethyl)thioureido)ethylthio)-2,3,4-tri-*O*-hexanoyl-α-D-glucopyranoside (49). To a solution of **34** (0.12 g, 0.20 mmol) and Et_3N (56 μL, 0.4 mmol) in DCM (6 mL), 2-[*N*,*N*-bis(2-(*N*-*tert*-butoxyaminocarbonyl)ethylamino]ethyl isothiocyanate **167** (0.09 g, 0.24 mmol) was added and the reaction mixture was stirred, under Ar atmosphere, at rt for 48 h. The reaction mixture was washed with aqueous diluted HCl (2 x 20 mL), dried ($MgSO_4$), filtered and concentrated. The residue was purified by column chromatography

(1:1→ 3:1 EtOAc-cyclohexane). Yield: 0.08 g (50%). R_f = 0.20 (1:1 EtOAc-cyclohexane); $[\alpha]_D$ = + 71.6 (*c* 1.0, DCM); IR: ν_{max} = 2959, 1748, 1685 $cm^{-1.}$

^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.46, 6.96 (2 bs, 2 H, N*H*CS), 5.46 (t, 1 H, $J_{2,3} = J_{3,4}$ = 9.8 Hz, H-3), 4.96 (t, 1 H, $J_{4,5}$ = 9.8 Hz, H-4), 4.90 (d, 1 H, $J_{1,2}$ = 3.5 Hz, H-1), 4.88 (bs, 2 H, N*H*Boc), 4.84 (dd, 1 H, H-2), 3.93 (bd, 1 H, H-5), 3.77 (q, 2 H, $J_{H,H}$ = 7.0 Hz, NHC*H*$_2$CH$_2$N), 3.52 (bs, 2 H, SCH$_2$C*H*$_2$N), 3.42 (s, 3 H, OCH_3), 3.11 (q, 4 H, NCH$_2$C*H*$_2$NHBoc), 2.82 (t, 2 H, $J_{H,H}$ = 7.0 Hz, NHCH$_2$C*H*$_2$N), 2.77-2.56 (m, 4 H, H-6a, H-6b, SC*H*$_2$), 2.50 (bs, 4 H, NC*H*$_2$CH$_2$NHBoc), 2.37-2.1 (m, 6 H, H-2$_{Hex}$), 1.57 (m, 6 H, $J_{H,H}$ = 7.0 Hz, H-3$_{Hex}$), 1.43 (s, 9 H, CMe_3), 1.27 (m, 12 H, H-4$_{Hex}$, H-5$_{Hex}$), 0.87 (t, 9 H, $J_{H,H}$ = 7.0 Hz, H-6$_{Hex}$).

^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 182.1 (CS), 172.0-171.0 (CO ester), 155.2 (CO carbamate), 96.6 (C-1), 70.9 (C-4), 70.1 (C-2), 69.9 (C-5), 69.6 (C-3), 55.8 (NCH$_2$CH$_2$NHBoc), 55.5 (OMe), 54.0 (SCH$_2$), 44.1 (NHCH$_2$CH$_2$N), 42.4 (SCH$_2$CH$_2$N), 39.4 (NCH$_2$CH$_2$NHBoc), 33.1 (C-6), 32.6 (NHCH$_2$CH$_2$N), 34.1 (C-2$_{Hex}$), 31.2 (C-5 $_{Hex}$), 28.4 (C*Me*$_3$) 24.5 (C-3$_{Hex}$), 22.1 (C-5$_{Hex}$), 13.9 (C-6$_{Hex}$).

ESIMS: *m/z* = 958.6 $[M + Na]^+$ Anal. Calcd for $C_{44}H_{81}N_5O_{12}S_2$: C, 56.44; H, 8.72; N, 7.48; S, 6.85. Found: C, 56.61; H, 8.89; N, 7.21; S, 6.60.

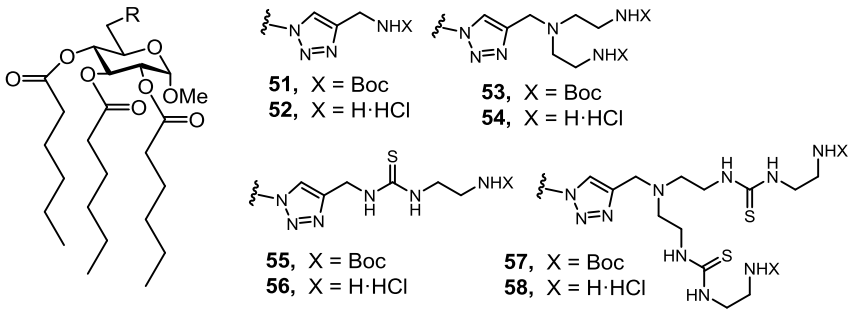
Methyl 6-(2-(N'-(2-(*N*,*N*-bis-(2-aminoethyl)amino)ethyl)thioureido)-ethylthio)-2,3,4-tri-*O*-hexanoyl-α-D-glucopyranoside trihydrochloride (50). Compound **50** was obtained by treatment of **49** (0.15 g, 0.16 mmol) with 1:1 TFA-DCM (2 mL) and freeze-drying from 10:1 H_2O-0.1 N HCl. Yield: 0.13 g (quantitative). $[\alpha]_D$ = + 46.8 (*c* 0.85, MeOH); IR: ν_{max} = 2958, 1747, 1675 cm^{-1}.

^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.98 (bs, 4 H, NHCS, NH_2HCl), 7.60 (bs, 1 H, NHCS), 5.47 (t, $J_{2,3} = J_{3,4}$ = 9.5 Hz, 1 H, H-3), 4.99 (t, 1 H, $J_{4,5}$ = 9.5 Hz, H-4), 4.91 (dd, 1 H, $J_{1,2}$ = 4 Hz, H-1), 4.85 (dd, 1 H, H-2), 3.96 (td, 1 H, $J_{5,6b}$ = 7.3 Hz, $J_{5,6a}$ = 2.4 Hz, H-5), 3.73 (bs, 2 H, SCH$_2$C*H*$_2$NHCS), 3.68 (bs, 2 H, NHCSC*H*$_2$CH$_2$N), 3.41 (s, 3 H, OCH_3), 3.11 (bs, 4 H, C*H*$_2$NH$_3$Cl), 2.84 (bs, 6 H, C*H*$_2$CH$_2$NH$_3$Cl, SC*H*$_2$CH$_2$NHCS), 2.75 (m, 3 H,

H-6a, NHCSCH$_2$C*H*$_2$N), 2.66 (dd, 1 H, $J_{6a,6b}$ = 13.8 Hz, H-6b), 2.32-2.19 (m, 6 H, H-2$_{Hex}$), 1.55 (m, 6 H, H-3$_{Hex}$), 1.27 (m, 12 H, H-4$_{Hex}$, H-5$_{Hex}$), 0.87 (m, 9 H, H-6$_{Hex}$).

^{13}C NMR (75 MHz, $CDCl_3$): δ = 173.1, 173.0, 172.6 (CO), 96.6 (C-1), 71.2 (C-4), 70.8 (C-2), 69.6 (C-3), 69.1 (C-5), 55.4 (OCH_3), 53.4 (NHCSCH$_2$*C*H$_2$N), 52.0 (*C*H$_2$CH$_2$NH$_2$HCl), 43.1 (SCH$_2$*C*H$_2$NHCS), 41.7 (NHCS*C*H$_2$CH$_2$N), 37.6 (*C*H$_2$NH$_3$Cl), 34.1, 34.0 (C-2$_{Hex}$), 33.0 (C-6), 32.7 (S*C*H$_2$CH$_2$NHCS), 31.2, 31.1 (C-4$_{Hex}$), 24.5, 24.4 (C-3$_{Hex}$), 22.2 (C-5$_{Hex}$), 13.8 (C-6$_{Hex}$).

ESIMS: *m/z* = 736.4 [M]$^+$. Anal. Calcd for $C_{34}H_{65}N_5O_8S_2 \cdot 3HCl$: C, 48.30; H, 8.11; N, 8.28; S, 7.58. Found: C, 48.24; H, 8.39; N, 8.15; S, 7.41.



Methyl 6-(4-*tert*-butoxycarbonylaminomethyl-1*H*-1,2,3-triazol-1-yl)-6-deoxy-2,3,4-tri-*O*-hexanoyl-α-D-glucopyranoside (51). To a solution of **35** (600 mg, 1.17 mmol) and (*N-tert*-butoxycarbonyl) propargylamine **170** (199 mg, 1.28 mmol) in H_2O-^{t}BuOH (9:1, 35 mL), **Si-BPA·Cu**$^+$ (35 mg) was added and the reaction mixture was stirred for 36 h at 85 ºC. The catalyst was filtered off and the solvent was removed. The residue was purified by column chromatography (1:2 → 3:1 EtOAc-cyclohexane). Yield: 667 mg (85%). R_f = 0.34 (1:2 EtOAc-cyclohexane); $[\alpha]_D$ = +65.01 (*c* 1.0, DCM); IR: ν_{max} = 2958, 1748, 1711, 733 cm^{-1}.

^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.62 (s, 1 H, =CH), 5.49 (t, 1 H, $J_{2,3}$ = $J_{3,4}$ = 9.8 Hz, H-3), 5.11 (bs, 2 H, NHBoc), 4.86 (d, 1 H, $J_{1,2}$ = 3.7 Hz, H-1), 4.85 (t, 1 H, $J_{4,5}$ = 9.8 Hz,

H-4), 4.80 (dd, 1 H, H-2), 4.52 (dd, 1 H, $J_{6a,6b}$ = 14.0 Hz, $J_{5,6a}$ = 2.5 Hz, H-6a), 4.36 (d, 2 H, $J_{H,H}$ = 6.1 Hz, CH_2 triazole), 4.28 (dd, 1 H, $J_{5,6b}$ = 8.8 Hz, H-6b), 4.13 (ddd, 1 H, H-5), 3.08 (s, 3 H, OCH_3), 2.26 (m, 6 H, H-2_{Hex}), 1.56 (m, 6 H, H-3_{Hex}), 1.41 (s, 9 H, CMe_3), 1.27 (m, 12 H, H-4_{Hex}, H-5_{Hex}), 0.87 (m, 9 H, H-6_{Hex}).

^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 174.2-173.2 (CO ester), 155.7 (CO carbamate), 145.5 (C-4 triazole), 123.5 (C-5 triazole), 94.1 (C-1), 79.7 (*C*Me_3), 70.6 (C-2), 69.7 (C-4), 69.2 (C-3), 68.1 (C-5), 55.9 (OMe), 50.9 (C-6), 36.2 (CH_2NHBoc), 34.3 (C-2_{Hex}), 30.7 (C-4_{Hex}), 28.3 (C*Me*$_3$), 24.2 (C-3_{Hex}), 22.6 (C-5_{Hex}), 13.9 (C-6_{Hex}).

ESIMS: *m/z* = 691.4 $[M + Na]^+$. Anal. Calcd for $C_{33}H_{56}N_4O_{10}$: C, 59.26; H, 8.44; N, 8.38. Found: C, 59.35; H, 8.27; N, 8.50.

Methyl 6-*(*4-aminomethyl-1*H*-1,2,3-triazol-1-yl)-6-deoxy-2,3,4-tri-*O*-hexanoyl-α-D-glucopyranoside hydrochloride (52). Treatment of **51** (501 mg, 0.75 mmol) with 1:1 TFA-DCM (6 mL) as indicated in general methods yielded **52**. Yield: 457 mg (quantitative). $[\alpha]_D$ = +99.29 (*c* 1.0, MeOH); IR: ν_{max} = 2957, 1757, 1678, 734 cm^{-1}.

^{1}H NMR (300 MHz, CD_3OD): δ = 8.58 (bs, 2 H, NH_2), 8.00 (s, 1 H, =CH), 5.48 (t, 1 H, $J_{2,3}$ = $J_{3,4}$ = 10.0 Hz, H-3), 4.90-4.76 (m, 3 H, H-1, H-2, H-4), 4.53 (dd, 1 H, $J_{5,6a}$= 2.7 Hz, $J_{6a,6b}$ = 14.5 Hz, H-6a), 4.42 (dd, 1 H, $J_{5,6b}$ = 7.7 Hz, H-6b), 4.34 (s, 1 H, CH_2-triazole), 4.15 (ddd, 1 H, $J_{4,5}$ = 10.0 Hz, H-5), 4.32 (s, 2 H, C*H*$_2$NH), 3.11 (s, 3 H, OCH_3), 2.45-2.12 (m, 6 H, H-2_{Hex}), 1.53 (m, 6 H, H-3_{Hex}), 1.25 (m, 12 H, H-4_{Hex}, H-5_{Hex}), 0.87 (m, 9 H, H-6_{Hex}).

^{13}C NMR (75.5 MHz, CD_3OD): δ = 173.2-172.3 (CO), 140.2 (C-4 triazole), 125.8 (C-5 triazole), 96.7 (C-1), 70.7 (C-2), 69.5 (C-4), 69.4 (C-3), 67.6 (C-5), 55.6 (OMe), 50.9 (C-6), 34.2 (CH_2-triazole), 33.9 (C-2_{Hex}), 31.3 (C-4_{Hex}), 24.5 (C-3_{Hex}), 22.1 (C-5_{Hex}), 13.7 (C-6_{Hex}).

ESIMS: *m/z* = 569.3 $[M + H]^+$; 1137.5 [2M]. Anal. Calcd for $C_{28}H_{49}ClN_4O_8$: C, 55.57; H, 8.16; N, 9.26. Found: C, 55.37; H, 8.00; N, 9.11.

Methyl 6-(4-(2,2-bis-*tert*-butoxycarbonylamino)ethylaminomethyl)-1*H*-1,2,3-triazol-1-yl-6-deoxy)-2,3,4-tri-*O*-hexanoyl-α-D-glucopyranoside (53). To a solution of **35** (0.20 g, 0.39 mmol) and 3-bis[2-*tert*-butoxycarbonylamino)ethyl]propargylamine **169** (0.29 g, 0.85 mmol) in 9:1 H_2O-^{t}BuOH (15 mL), **Si-BPA·Cu$^+$** (0.02 g) was added and the reaction mixture was refluxed for 36 h at 85 ºC. The catalyst was filtered off and the solvent was removed. The residue was purified by column chromatography (1:1 → 2:1 EtOAc-cyclohexane). Yield: 0.25 g (78%). R_f = 0.61 (9:1 DCM-MeOH); $[\alpha]_D$ = +50.5 (*c* 1.0, DCM); IR: ν_{max} = 2957, 2359, 1748, 1703, 734 cm^{-1}.

^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.59 (s, 1 H, =CH), 5.49 (t, 1 H, $J_{2,3} = J_{3,4}$ = 10.3 Hz , H-3), 4.89 (bs, 2 H, N*H*Boc), 4.87 (d, 1 H, $J_{1,2}$ = 3.5 Hz, H-1), 4.85 (t, 1 H, $J_{4,5}$ = 10.3 Hz, H-4), 4.81 (dd, 1 H, H-2), 4.53 (dd, 1 H, $J_{6a,6b}$ = 14.0 Hz, $J_{5,6a}$ = 2.6 Hz, H-6a), 4.29 (dd, 1 H, $J_{5,6b}$ = 9.0 Hz, H-6b), 4.17 (ddd, 1 H, H-5), 3.80 (m, 2 H, CH_2-triazole), 3.18 (bs, 4 H, C*H*$_2$NHBoc), 3.07 (s, 3 H, OCH_3), 2.55 (t, 4 H, C*H*$_2$CH$_2$NHBoc), 2.27 (m, 6 H, H-2$_{Hex}$), 1.58 (s, 9 H, CMe_3), 1.44 (m, 6 H, H-3$_{Hex}$), 1.30 (m, 12 H, H-4$_{Hex}$, H-5$_{Hex}$), 0.89 (m, 9 H, H-6$_{Hex}$).

^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 173.9, 173.5, 173,1 (CO ester), 156.2 (CO carbamate), 144.1 (C-4 triazole), 124.3 (C-5 triazole), 91.4 (C-1), 78.1 (*C*Me$_3$), 70.6 (C-2), 69.8 (C-4), 69.3 (C-3), 68.0 (C-5), 53.1 (*C*H$_2$CH$_2$NHBoc), 50.6 (C-6), 48.3 (CH_2 triazole), 38.4 (*C*H$_2$NHBoc), 34.1 (C-2$_{Hex}$), 31.7 (C-4$_{Hex}$), 28.5 (C*Me$_3$*), 24.6 (C-3$_{Hex}$), 22.6 (C-5$_{Hex}$), 13.8 (C-6$_{Hex}$).

ESIMS: *m/z* = 877.5 [M + Na]$^+$. Anal. Calcd for $C_{42}H_{74}N_6O_{12}$: C, 59.00; H, 8.72; N, 9.83. Found: C, 59.09; H, 8.77; N, 9.64.

Methyl 6-deoxy-6-(4-(2,2-diaminoethylaminomethyl)-1*H*-1,2,3-triazol-1-yl)-2,3,4-tri-*O*-hexanoyl-α-D-glucopyranoside dihydrochloride (54). Compound **54** was obtained by treatment of **53** (0.42 g, 0.49 mmol) with 1:1 TFA-DCM (5 mL) and freeze-

drying from 10:1 H_2O-0.1 N HCl. Yield: 0.35 g (quantitative). $[\alpha]_D$ = +38.1 (*c* 1.0, MeOH); IR: ν_{max} = 2957, 1748, 1675 $cm^{-1.}$

^1H NMR (300 MHz, CD_3OD): δ = 8.07 (s, 1 H, =CH), 5.42 (t, 1 H, $J_{2,3}$ = $J_{3,4}$ = 9.5 Hz, H-3), 4.93 (d, 1 H, $J_{1,2}$ = 3.5 Hz, H-1), 4.82 (dd, 1 H, H-2), 4.76 (t, 1 H, $J_{4,5}$ = 9.5 Hz, H-4), 4.64 (m, 2 H, H-6a, H-6b), 4.27 (ddd, 1 H, $J_{5,6a}$ = 3.7 Hz, $J_{5,6b}$ = 6.0 Hz, H-5), 3.92 (s, 2 H, CH_2 triazole), 3.26 (s, 3 H, OMe), 3.16 (t, 4 H, $J_{H,H}$ = 6.4 Hz, $CH_2CH_2NH_2$), 2.82 (t, 4 H, $CH_2CH_2NH_2$), 2.48-2.15 (m, 6 H, H-2_{Hex}), 1.58 (m, 6 H, H-3_{Hex}), 1.33 (m, 12 H, H-4_{Hex}, H-5_{Hex}), 0.92 (m, 9 H, H-6_{Hex}).

^{13}C NMR (75.5 MHz, CD_3OD): δ = 174.2-173.9 (CO), 143.8 (C-4 triazole), 126.9 (C-5 triazole), 98.6 (C-1), 71.8 (C-2), 70.7 (C-3), 70.3 (C-4), 68.9 (C-5), 56.0 (OCH_3), 52.0 ($CH_2CH_2NH_2$), 51.6 (C-6), 47.4 (CH_2 triazole), 38.2 ($CH_2CH_2NH_2$), 34.9, 34.8, 34.7 (C-2_{Hex}), 32.4, 32.2 (C-4_{Hex}), 25.6, 25.5 (C-3_{Hex}), 23.4 (C-5_{Hex}), 14.2 (C-6_{Hex}).

ESIMS: *m/z* = 831.3 $[M + TFA + Cl + Cu]^+$; 717.3 $[M + Cu + Cl]^+$; Anal. Calcd for $C_{32}H_{59}N_6O_8 \cdot 2HCl$: C, 52.81; H, 8.31; N, 11.55. Found: C, 52.69; H, 8.1; N, 11.72.

Methyl 6-(4-(2-*N*'-(2-(*N*-*tert*-butoxycarbonyl)aminoethyl)thioureido)-methyl-1*H*-1,2,3-triazol-1-yl)-6-deoxy-2,3,4-tri-*O*-hexanoyl-α-D-glucopyranoside (55). To a solution of **52** (200 mg, 0.33 mmol) and Et_3N (68.6 μL, 0.49 mmol) in DCM (10 mL), *tert*-butyl *N*-(2-isothiocyanoethyl)carbamate **168** (101 mg, 0.49 mmol) was added and the reaction mixture was stirred overnight at rt. The reaction mixture was washed with aqueous diluted HCl (3 x 10 mL) and the organic phase was dried ($MgSO_4$), filtered, and concentrated. The residue was purified by column chromatography (1:1 → 3:2 EtOAc-cyclohexane). Yield: 195 mg (76%). R_f = 0.38 (2:1 EtOAc-cyclohexane); $[\alpha]_D$ = +47.19 (*c* 1.0, DCM); UV (DCM): λ_{max} = 250 nm (ε_{mM} 21.2); IR: ν_{max} = 2957, 1748, 1705 cm^{-1}.

^1H NMR (300 MHz, $CDCl_3$): δ = 7.81 (s, 1 H, =CH), 7.19, 7.07 (bs, 2 H, CH_2NHCS), 5.48 (t, 1 H, $J_{2,3}$ = $J_{3,4}$ = 10.0 Hz, H-3), 5.08 (bs, 1 H, NHBoc), 4.88 (d, 1 H, $J_{1,2}$ = 3.7 Hz, H-1), 4.82 (t, 1 H, $J_{4,5}$ = 9.7 Hz, H-4), 4.80 (dd, 1 H, H-2), 4.77 (bs, 2 H, CH_2 triazole),

4.53 (dd, 1 H, $J_{6a,6b}$ = 14.1 Hz, $J_{5,6a}$ = 2.3 Hz, H-6a), 4.36 (dd, 1 H, $J_{5,6b}$ = 8.1 Hz, H-6b), 4.13 (ddd, 1 H, H-5), 3.62 (bs, 2 H, C*H*$_2$CH$_2$NHBoc), 3.29 (q, 2 H, $J_{NH,H}$ = $J_{H,H}$ = 5.8 Hz, CH$_2$C*H*$_2$NHBoc), 3.12 (s, 3 H, OCH$_3$), 2.26 (m, 6 H, H-2$_{Hex}$), 1.55 (m, 6 H, H-3$_{Hex}$), 1.40 (s, 9 H, CMe$_3$), 1.26 (m, 12 H, H-4$_{Hex}$, H-5$_{Hex}$), 0.86 (m, 9 H, H-6$_{Hex}$).

^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 183.0 (CS), 172.9-172.5 (CO ester), 144.5 (C-4 triazole), 124.2 (C-5 triazole), 96.7 (C-1), 79.9 (*C*Me$_3$), 70.6 (C-2), 69.6 (C-4), 69.3 (C-3), 67.9 (C-5), 56.1 (OMe), 50.9 (C-6), 45.1 (*C*H$_2$CH$_2$NHBoc), 40.0 (*C*H$_2$NHBoc), 39.2 (*C*H$_2$-triazole), 34.2 (C-2$_{Hex}$), 31.4 (C-4$_{Hex}$), 28.3 (C*Me*$_3$), 24.9 (C-3$_{Hex}$), 22.3 (C-5$_{Hex}$), 14.1 (C-6$_{Hex}$).

ESIMS: *m/z* = 793.2 $[M + Na]^+$; 1563.5 $[2 M + Na]^+$. Anal. Calcd for $C_{36}H_{62}N_6O_{10}$: C, 56.08; H, 8.11; N, 10.9; S, 4.16. Found: C, 56.02; H, 7.88; N, 10.81; S, 4.12.

Methyl 6-(4-(2-*N*'-(2-aminoethylthioureido)methyl-1*H*-1,2,3-triazol-1-yl)-6-deoxy-2,3,4-tri-*O*-hexanoyl-α-D-glucopyranoside hydrochloride (56). Treatment of **55** (190 mg, 0.246 mmol) with 1:1 TFA-DCM (2 mL) as indicated in general methods yielded **56**. Yield: 167 mg (96%). $[\alpha]_D$ = +56.2 (*c* 1.0, MeOH); UV (MeOH): λ_{max} = 244 nm (ε_{mM} 9.0); IR: ν_{max} = 2955, 1746 cm^{-1}.

^{1}H NMR (300 MHz, CD_3OD): δ = 8.01 (s, 1 H, =CH), 5.43 (t, 1 H, $J_{2,3}$ = $J_{3,4}$ = 9.7 Hz, H-3), 4.91 (d, 1 H, $J_{1,2}$ = 4.2 Hz, H-1), 4.82 (m, 4 H, H-4, H-2, CH$_2$-triazole), 4.58 (m, 2 H, H-6a, H-6b), 4.18 (ddd, 1 H, $J_{4,5}$ = 10.0 Hz, $J_{5,6a}$ = 4.4 Hz, $J_{5,6b}$ = 5.3 Hz, H-5), 3.87 (t, 2 H, $J_{H,H}$ = 5.8 Hz, C*H*$_2$CH$_2$NH$_2$), 3.19 (s, 3 H, OCH$_3$), 3.18 (t, 2 H, CH$_2$C*H*$_2$NH$_2$), 2.32 (m, 6 H, H-2$_{Hex}$), 1.58 (m, 6 H, H-3$_{Hex}$), 1.31 (m, 12 H, H-4$_{Hex}$, H-5$_{Hex}$), 0.91 (m, 9 H, H-6$_{Hex}$)

^{13}C NMR (75.5 MHz, CD_3OD): δ = 174.2-173.8 (CO), 143.6 (C-4 triazole), 124.9 (C-5 triazole), 98.1 (C-1), 71.8 (C-2), 71.2 (C-3), 70.8 (C-4), 69.1 (C-5), 56.1 (OMe), 51.7 (C-6), 42.4 (*C*H$_2$CH$_2$NH$_2$), 40.9 (CH$_2$*C*H$_2$NH$_2$), 40.3 (CH$_2$ triazole), 34.5 (C-2$_{Hex}$), 31.9 (C-4$_{Hex}$), 25.5 (C-3$_{Hex}$), 23.4 (C-5$_{Hex}$), 14.1 (C-6$_{Hex}$).

ESIMS: *m/z* = 671 $[M + H]^+$. Anal. Calcd for $C_{31}H_{55}ClN_6O_8S$: C, 52.64; H, 7.84; N, 11.88; S, 4.53. Found: C, 52.52; H, 7.64; N, 12.03, 4.37.

Dendritic Boc-protected diaminoethyl-bis(thiourea) glucopyranoside derivative (57). To a solution of **54** (0.20 g, 0.27 mmol) and Et_3N (115 μL, 0.82 mmol) in DCM (12 mL), *tert*-butyl *N*-(2-isothiocyanoethyl)carbamate **168** (0.17 mg, 0.82 mmol) was added and the mixture was stirred overnight at rt. The reaction mixture was washed with aqueous diluted HCl (3 x 10 mL) and the organic phase was dried ($MgSO_4$), filtered, and concentrated. The residue was purified by column chromatography (3:1 EtOAc-cyclohexane → 20:1 DCM-MeOH). Yield: 0.15 g (52%). R_f = 0.44 (9:1 DCM-MeOH); $[\alpha]_D$ = + 31.7 (*c* 1.0, DCM); UV (DCM): λ_{max} = 248 nm (ε_{mM} 47.8); IR: ν_{max} = 2959, 1750, 1698, 736 cm^{-1}.

^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.62 (s, 1 H, =CH), 7.18, 6.94 (bs, 4 H, N*H*CS), 5.49 (t, 1 H, $J_{2,3} = J_{3,4}$ = 9.5 Hz , H-3), 5.38 (bs, 2 H, NHBoc), 4.82 (d, 1 H, $J_{1,2}$ = 3.5 Hz, H-1), 4.80 (t, 1 H, $J_{4,5}$ = 9.5 Hz, H-4), 4.80 (dd, 1 H, H-2), 4.53 (dd, 1 H, $J_{6a,6b}$ = 14.5 Hz, $J_{5,6a}$ = 2.6 Hz, H-6a), 4.37 (dd, 1 H, $J_{5,6b}$ = 8.0 Hz, H-6b), 4.17 (ddd, 1 H, H-5), 3.79 (s, 2 H, CH_2 triazole), 3.62 (bs, 4 H, C*H*$_2$CH$_2$NHBoc), 3.54 (bs, 4 H, NCH$_2$C*H*$_2$NHCS), 3.31 (m, 4 H, C*H*$_2$NHBoc), 3.12 (s, 1 H, OCH_3), 2.69 (bs, 4 H, NC*H*$_2$CH$_2$NHCS), 2.42-2.13 (m, 6 H, H-2$_{Hex}$), 1.55 (m, 6 H, H-3$_{Hex}$), 1.42 (s, 18 H, CMe_3), 1.26 (m, 12 H, H-4$_{Hex}$, H-5$_{Hex}$), 0.87 (t, 9 H, $J_{H,H}$ = 6.0 Hz, H-6$_{Hex}$).

^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 182.7 (CS), 173.0-172.5 (CO ester), 155.5 (CO carbamate), 144.5 (C-4 triazole), 124.4 (C-5 triazole), 96.7 (C-1), 79.8 (*C*Me$_3$), 70.2 (C-2), 69.6 (C-4), 69.2 (C-3), 67.8 (C-5), 55.4 (OMe), 52.4 (NCH$_2$CH$_2$NHCS), 50.7 (C-6), 48.0 (CH_2 triazole), 44.6 (*C*H$_2$CH$_2$NHBoc), 42.1 (NCH$_2$*C*H$_2$NHCS), 40.1 (CH$_2$NHBoc), 34.3 (C-2$_{Hex}$), 30.8 (C-4$_{Hex}$), 28.1 (C*Me*$_3$), 24.2 (C-3$_{Hex}$), 22.6 (C-5$_{Hex}$), 14.1 (C-6$_{Hex}$).

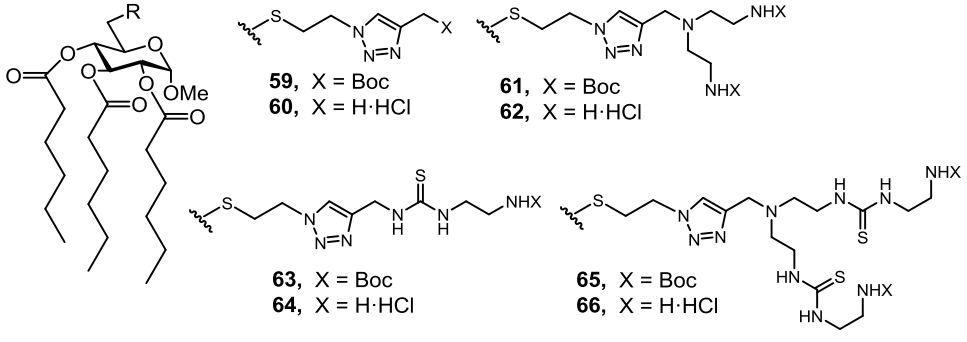
ESIMS: *m/z* = 1081.5 $[M + Na]^+$. Anal. Calcd for $C_{48}H_{86}N_{10}O_{12}S_2$: C, 54.42; H, 8.18; N, 13.22; S, 6.05. Found: C, 54.37; H, 7.98; N, 13.28; S, 5.85.

Dendritic diaminoethyl-bis(thiourea) glucopyranoside dihydrochloride derivative (58). Compound **58** was obtained by treatment of **57** (0.12 g, 0.12 mmol) with 1:1 TFA-DCM (2 mL) and freeze-drying from 10:1 H_2O-0.1 N HCl solution. Yield: 0.10 g (91%). $[\alpha]_D$ = +47.7 (*c* 1.0, MeOH); UV (MeOH): λ_{max} = 244 nm (ε_{mM} 29.1); IR: ν_{max} = 2955, 1748, 1676 cm^{-1}.

^{1}H NMR (300 MHz, CD_3OD): δ = 8.43 (s, 1 H, =CH), 5.43 (t, 1 H, $J_{2,3} = J_{3,4}$ = 9.7 Hz, H-3), 4.97 (d, 1 H, $J_{1,2}$ = 3.5 Hz, H-1), 4.90 (dd, 1 H, H-2), 4.82 (t, 1 H, $J_{4,5}$ = 9.7 Hz, H-4), 4.77 (m, 2 H, CH_2 triazole), 4.70 (m, 2 H, H-6a, H-6b), 4.27 (ddd, 1 H, $J_{5,6a}$ = 3.7 Hz, $J_{5,6b}$ = 5.6 Hz, H-5), 4.06 (bs, 4 H, C*H*$_2$CH$_2$NH$_2$), 3.87 (t, 4 H, $J_{H,H}$ = 5.8 Hz, NCH$_2$C*H*$_2$NHCS), 3.52 (t, 4 H, $J_{H,H}$ = 5.8 Hz, C*H*$_2$NH$_2$), 3.25 (s, 3 H, OCH_3), 3.21 (t, 4 H, $J_{H,H}$ = 6.0 Hz, NC*H*$_2$CH$_2$NHCS), 2.50-2.15 (m, 6 H, H-2$_{Hex}$), 1.56 (m, 6 H, H-3$_{Hex}$), 1.31 (m, 12 H, H-4$_{Hex}$, H-5$_{Hex}$), 0.91 (m, 9 H, H-6$_{Hex}$).

^{13}C NMR (75.5 MHz, CD_3OD): δ = 184.1 (CS), 174.3-173.9 (CO), 137.2 (C-4 triazole), 130.1 (C-5 triazole), 98.2 (C-1), 71.8 (C-2), 71.2 (C-3), 70.5 (C-4), 68.7 (C-5), 56.2 (OMe), 54.4 (CH_2NH_2), 51.4 (C-6), 48.4 (CH_2-triazole), 42.2 ($CH_2CH_2NH_2$), 40.4 (N*C*H$_2$CH$_2$NHCS), 40.0 (CH_2NH_2), 34.4 (C-2$_{Hex}$), 32.0 (C-4$_{Hex}$), 25.2 (C-3$_{Hex}$), 22.8 (C-5$_{Hex}$), 14.0 (C-6$_{Hex}$).

ESIMS: *m/z* = 859.5 $[M + Na]^+$; 921.4 $[M + Cu]^+$. Anal. Calcd for $C_{38}H_{72}Cl_2N_{10}O_8S_2$: C, 48.97; H, 7.79; N, 15.03; S, 6.88. Found: C, 48.71; H, 77.40; N, 15.23; S, 6.65.



Methyl 6-(2-(4-*tert*-butoxycarbonylaminomethyl-1*H*-1,2,3-triazol-1-yl)ehtylthio)-6-deoxy-2,3,4-tri-*O*-hexanoyl-α-D-glucopyranoside (59). To a solution of 1**4** (200 mg, 0.34 mmol) and *N*-(*tert*-butoxycarbonyl)propargylamine **170** (63 mg, 0.52 mmol) in 9:1 ^{t}BuOH:H_2O mixture (10 mL), **Si-BPA·Cu^{+}** (12 mg) was added and the reaction mixture was refluxed for 24 h at 85 ºC. The catalyst was filtered off, the solvent was removed, and the residue was purified by column chromatography (1:1 → 1:0 EtOAc-cyclohexane). Yield: 241 mg (95%). $[\alpha]_D$ = + 66.4 (*c* 1.0, DCM); IR: ν_{max} = 2957, 2931, 1747, 1714 cm^{-1}.

^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.57 (s, 1 H, =CH), 5.45 (t, 1 H, $J_{2,3} = J_{3,4}$ = 9.5 Hz, H-3), 5.16 (bs, 1 H, NHBoc), 4.94 (t, 1 H, $J_{4,5}$ = 9.6 Hz, H-4), 4.89 (d, 1 H, $J_{1,2}$ = 3.7 Hz, H-1), 4.82 (dd, 1 H, H-2), 4.49 (t, 2 H, $J_{H,H}$ = 6.7, CH_2N), 4.37 (d, 2 H, $J_{H,H}$ = 6.0 Hz, CH_2triazole), 3.89 (dt, 1 H, $J_{5,6}$ = 5.4 Hz, H-5), 3.39 (s, 3 H, OCH_3), 3.08 (m, 2 H, C*H*$_2$S), 2.52 (d, 2 H, H-6), 2.35-2.14 (m, 6 H, H-2$_{Hex}$), 1.55 (m, 6 H, H-3$_{Hex}$), 1.43 (s, 9 H, CMe_3), 1.26 (m, 12 H, H-4$_{Hex}$, H-5$_{Hex}$), 0.86 (m, 9 H, H-6$_{Hex}$).

^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 173.0-172.0 (CO ester), 155.8 (CO carbamate), 145.5 (C4 triazole), 122.3 (C5 triazole), 96.6 (C-1), 79.7 (*C*Me_3), 71.2 (C-4), 70.8 (C-2), 69.8 (C-5), 69.4 (C-3), 55.4 (OMe), 50.1 (CH_2Ntriazole), 36.1 (CH_2-triazole), 34.1 (C-2$_{Hex}$), 33.5 (CH_2S), 33.4 (C-6), 31.1 (C-4$_{Hex}$), 28.4 (C*Me*$_3$), 24.4 (C-3$_{Hex}$), 22.2 (C-5$_{Hex}$), 13.8 (C-6$_{Hex}$).

ESIMS: *m/z* = 1791.3 $[M + Cu]^+$ 1519.7 $[2M + Cu]^+$. Anal. Calcd for $C_{35}H_{60}N_4O_{10}S$: C, 57.67; H, 8.30; N, 7.69; S, 4.40. Found: C, 57.61; H, 8.33; N, 7.56; S, 4.21.

Methyl 6-(2-(4-aminomethyl-1*H*-1,2,3-triazol-1-yl)ehtylthio)-6-deoxy-2,3,4-tri-*O*-hexanoyl-α-D-glucopyranoside hydrochloride (60). Treatment of **59** (221 mg, 0.303 mmol) with 1:1 TFA-DCM (1 mL) as described in general methods yielded **60**. Yield: 185 mg (91%). $[\alpha]_D$ = + 56.56 (*c* 1.0, MeOH); IR: ν_{max} = 2956, 2930, 1746, 1679 cm^{-1}.

^{1}H NMR (300 MHz, CD_3OD): δ = 8.1 (s, 1 H, =CH), 5.41 (t, 1 H, $J_{2,3} = J_{3,4}$ = 9.6 Hz, H-3), 5.00 (t, 1 H, $J_{4,5}$ = 9.6 Hz, H-4), 4.92 (d, 1 H, $J_{1,2}$ = 3.7 Hz, H-1), 4.84 (dd, 2 H, H-

2), 4.65 (t, 2 H, $J_{H,H}$ = 6.5 Hz, CH_2N triazole), 4.26 (s, 2 H, CH_2 triazole), 3.93 (ddd, 1 H, $J_{5,6a}$ = 7.54 Hz, $J_{5,6b}$ = 10.5 Hz, H-5), 3.43 (s, 3 H, OMe), 3.16 (m, 2 H, CH_2S), 2.70 (dd, 1 H, $J_{6a,6b}$ = 14.5 Hz, H-6a), 2.60 (dd, 1 H, H-6b), 2.41-2.17 (m, 6 H, H-2_{Hex}), 1.57 (m, 6 H, H-3_{Hex}), 1.30 (m, 12 H, H-4_{Hex}, H-5_{Hex}), 0.90 (t, 9 H, $J_{H,H}$ = 6.0 Hz, H-6_{Hex}).

^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 174.3-174.1, 174.0 (CO ester), 141.0 (C-4 triazole), 125.9 (C5 triazole), 97.8 (C-1), 72.4 (C-4), 72.1 (C-2), 71.2 (C-5), 71.1 (C-3), 55.9 (OMe), 51.2 (CH_2Ntriazole), 35.5 (CH_2-triazole), 34.9 (C-2_{Hex}), 34.3 (CH_2S), 34.0 (C-6), 32.4 (C-4_{Hex}), 25.6 (C-3_{Hex}), 23.3 (C-5_{Hex}), 14.2 (C-6_{Hex}).

ESIMS: *m/z* = 651.3 $[M + Na]^+$, 663.1 $[M + Cl]^-$. Anal. Calcd for $C_{30}H_{53}ClN_4O_8S$: C, 54.16; H, 8.03; Cl, 5.33; N, 8.42; S, 4.82. Found: C, 53.94; H, 7.79; N, 8.31; S, 4.59.

Methyl 6-(2-(4-(2,2-bis-*tert*-butoxycarbonylamino)ethylaminomethyl)-1*H*-1,2,3-triazol-1-yl-6-deoxy)ehtylthio)-2,3,4-tri-*O*-hexanoyl-α-D-glucopyranoside (61). To a solution of **14** (200 mg, 0.365 mmol) and 3-bis[2-*tert*-butoxycarbonylamino)ethyl]propargylamine **169** (138 mg, 0.56 mmol) in 9:1 ^{t}BuOH:H_2O mixture (10 mL), **Si-BPA·Cu$^+$** (12 mg) was added and the reaction mixture was refluxed for 24 h at 85 ºC. The catalyst was filtered off and the solvent was removed. The residue was purified by column chromatography (2:1 → 4:1 → 1:0 EtOAc-cyclohexane). Yield: 292 mg (87%). $[\alpha]_D$ = + 55.5 (*c* 1.0, DCM); IR: ν_{max} = 2958, 1748, 1702 cm^{-1}.

^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.54 (s, 1 H, =CH), 5.45 (t, 1 H, $J_{2,3}$ = $J_{3,4}$ = 9.8 Hz, H-3), 5.04 (bs, 2 H, NHBoc), 4.99 (t, 1 H, $J_{4,5}$ = 9.6 Hz, H-4), 4.91 (d, 1 H, $J_{1,2}$ = 4.0 Hz, H-1), 4.82 (dd, 1 H, H-2), 4.50 (t, 2 H, $J_{H,H}$ = 7.0 Hz, CH_2N), 3.91 (m, 1 H, H-5), 3.79 (s, 2 H, C*H*$_2$triazole), 3.39 (s, 3 H, OMe), 3.19 (q, 4 H, $J_{H,H}$ = 5.8 Hz, C*H*$_2$NHBoc), 3.09 (m, 4 H, CH_2S), 2.56 (m, 6 H, C*H*$_2$$CH_2$NHBoc, H-6a, H-6b), 2.36-2.14 (m, 12 H, H-2_{Hex}), 1.54 (m, 12 H, H-3_{Hex}), 1.43 (s, 36 H, CMe_3), 1.25 (m, 24 H, H-4_{Hex}, H-5_{Hex}), 0.87 (m, 18 H, H-6_{Hex}).

^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 172.9, 172.5 (CO ester), 156.2 (CO carbamate), 144.2 (C4 triazole), 123.1 (C5 triazole), 96.6 (C-1), 79.2 (*C*Me_3), 71.1 (C-4), 70.8 (C-2),

69.9 (C-5), 69.4 (C-3), 55.4 (OMe), 53.2 (*C*H_2NHBoc), 50.0 (*C*H_2N triazole), 48.2 (CH_2-triazole), 38.3 (*C*$H_2$$CH_2$NHBoc) 33.5 (*C*$H_2$S), 34.1 (C-$2_{Hex}$), 33.3 (C-6), 33.1 (C-$4_{Hex}$), 28.4 ($CMe_3$), 24.5 (C-$3_{Hex}$), 22.2 (C-$5_{Hex}$), 13.8 (C-$6_{Hex}$).

ESIMS: *m/z* = 915.2 $[M + Na]^+$. Anal. Calcd for $C_{44}H_{78}N_6O_{12}S$: C, 57.74; H, 8.59; N, 9.18; S, 3.50. Found: C, 57.82; H, 8.51; N, 9.05; S, 3.39.

Methyl 6-deoxy-6-(2-(4-(2,2-diaminoethylaminomethyl)-1*H*-1,2,3-triazol-1-yl)ehtylthio)-2,3,4-tri-*O*-hexanoyl-α-D-glucopyranoside dihydrochloride (62). Compound **61** (290 mg, 0.316 mmol) was treated with 1:1 TFA:DCM (1 mL) at rt for 30 min. Then, the solvent was evaporated and acid traces were removed by co-evaporation with water. The residue was dissolved in 10:1 H_2O-HCl 0.1 N and freeze-dried to yield quantitatively compound **62.** Yield: 249 mg (quantitave). $[\alpha]_D$ = + 93.7 (*c* 1.0, MeOH); IR: ν_{max} = 2956, 1746, 1676, 721 cm^{-1}.

^{1}H NMR (300 MHz, CD_3OD): δ = 8.17 (s, 1 H, =CH), 5.41 (t, 1 H, $J_{2,3} = J_{3,4}$ = 10.0 Hz, H-3), 5.00 (t, 1 H, $J_{4,5}$ = 10.0 Hz, H-4), 4.93 (d, 1 H, $J_{1,2}$ = 4.0 Hz, H-1), 4.85 (dd, 1 H, H-2), 4.64 (t, 2 H, $J_{H,H}$ = 6.6 Hz, CH_2N), 4.01 (bs, 2 H, CH_2 triazole), 3.94 (ddd, 1 H, $J_{5,6b}$ = 7.2 Hz, $J_{5,6a}$ = 2.9 Hz, H-5), 3.44 (s, 3 H, OMe), 3.20 (m, 6 H, CH_2S, C*H*$_2$$NH_2$), 2.92 (t, 4 H, $J_{H,H}$ = 6.0 Hz, C*H*$_2$$CH_2$$NH_2$), 2.72 (dd, 1 H, $J_{6a,6b}$ = 14.5 Hz, H-6a), 2.61 (dd, 1 H, H-6b), 2.42-2.17 (m, 6 H, H-2_{Hex}), 1.55 (m, 6 H, H-3_{Hex}), 1.30 (m, 12 H, H-4_{Hex}, H-5_{Hex}), 0.90 (m, 9 H, H-6_{Hex}).

^{13}C NMR (75.5 MHz, CD_3OD): δ = 185.6 (CS), 174.3, 174.2, 174.0 (CO), 143.0 (C4 triazole), 126.5 (C5 triazole), 98.0 (C-1), 72.4 (C-4), 72.1 (C-2), 71.2 (C-5, C-3), 56.0 (OMe), 52.0 (CH_2NH_2), 51.3 (CH_2N triazole), 47.8 (CH_2-triazole), 38.0 (C*H*$_2$$CH_2$$NH_2$), 34.9 (C-$2_{Hex}$), 34.2* ($CH_2$S), 34.0* (C-6), 32.4 (C-4_{Hex}), 25.2 (C-3_{Hex}), 23.3 (C-5_{Hex}), 14.2 (C-6_{Hex}).

ESIMS: *m/z* = 777.1 $[M + Cu]^+$. Anal. Calcd for $C_{34}H_{64}Cl_2N_6O_8S$: C, 51.83; H, 8.19; N, 10.67; S, 4.07. Found: C, 51.63; H, 7.95; N, 10.33; S, 3.81.

Methyl 6-(2-(4-*tert*-butoxycarbonylaminomethyl-1*H*-1,2,3-triazol-1-yl)ehtylthio)-6-deoxy-2,3,4-tri-*O*-hexanoyl-α-D-glucopyranoside (63). To a solution of **60** (110 mg, 0.165 mmol) and Et_3N (35 μL, 0.248 mmol), *tert*-butyl *N*-(2-isothiocyanoethyl) carbamate **168** (50.2 mg, 0.248 mmol) was added, and the mixture was stirred at rt, under Ar atmosphere, for 48 h. The mixture was washed with HCl 0.1 N (2 x 10 mL) and the organic phase was dried ($MgSO_4$), filtered and concentrated. The residue was purified by column chromatography (1:2 → 1:1 → 2:1 EtOAc-cyclohexane). Yield: 124 mg (90%). $[\alpha]_D$ = + 67.63 (*c* 1.0, DCM); UV (DCM): λ_{max} = 250 nm (ε_{mM} 13.6); IR: ν_{max} = 2958, 2932, 1745, 1703, 1672, 1164, 906, 726 cm^{-1}.

^1H NMR (300 MHz, $CDCl_3$): δ = 8.00 (s, 1 H, =CH), 7.18, 7.03 (bs, 2 H, NHCS), 5.44 (t, 1 H, $J_{2,3} = J_{3,4}$ = 9.5 Hz, H-3), 5.11 (bs, 1 H, NHBoc), 4.92 (t, 1 H, $J_{4,5}$ = 9.5 Hz, H-4), 4.89 (d, 1 H, $J_{1,2}$ = 3.5 Hz, H-1), 4.82 (dd, 1 H, H-2), 4.78 (bs, 2 H, CH_2 triazole), 4.50 (t, 2 H, $J_{H,H}$ = 6.7 Hz, CH_2N), 3.87 (dt, 1 H, $J_{5,6}$ = 5.2 Hz, H-5), 3.63 (m, 2 H, NHCSC$H_2$$CH_2$NHBoc), 3.43 (s, 3 H, OMe), 3.30 (q, 2 H, $J_{H,H} = J_{H,NH}$ = 5.7 Hz, CH_2NHBoc), 3.08 (m, 2 H, CH_2S), 2.51 (d, 2 H, H-6), 2.36-2.12 (m, 6 H, H-2_{Hex}), 1.54 (m, 6 H, H-3_{Hex}), 1.40 (s, 9 H, CMe_3), 1.26 (m, 12 H, H-4_{Hex}, H-5_{Hex}), 0.86 (m, 18 H, H-6_{Hex}).

^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 173.0, 172.7, 173.6 (CO ester), 152.9 (CO carbamate), 144.5 (C4 triazole), 123.3 (C5 triazole), 96.6 (C-1), 79.8 (*C*Me_3), 71.3 (C-4), 70.8 (C-2), 69.8 (C-5), 69.4 (C-3), 55.4 (OMe), 50.3 (CH_2N), 39.4 (NCH_2*C*H_2NHCS), 36.4 (NHCS*C*$H_2$$CH_2$NHBoc), 35.5 ($CH_2$-triazole), 34.0 (C-$2_{Hex}$), 33.4 ($CH_2S$), 31.4 (C-6), 31.1 (C-$4_{Hex}$), 28.3 (C*Me*$_3$), 24.5 (C-$3_{Hex}$), 22.2 (C-$5_{Hex}$), 13.8 (C-$6_{Hex}$).

ESIMS: *m/z* = 853.4 $[M + Na]^+$. Anal. Calcd for $C_{38}H_{66}N_6O_{10}S_2$: C, 54.92; H, 8.00; N, 10.11; S, 7.72. Found: C, 55.13; H, 8.10; N, 10.03; S, 7.60.

Methyl 6-2-*(*4-aminomethyl-1*H*-1,2,3-triazol-1-yl)ethylthio)-6-deoxy-2,3,4-tri-*O*-hexanoyl-α-D-glucopyranoside hydrochloride (64). Treatment of **63** (108 mg, 0.13

mmol) with 1:1 TFA:DCM (1 mL) as described in general methods yielded **64**. Yield: 98 mg (98%). $[\alpha]_D$ = + 67.8 (*c* 1.0, MeOH); UV (MeOH): λ_{max} = 243 nm (ε_{mM} 11.2); IR: ν_{max} = 3319, 2955, 1748, 1678 cm^{-1}.

^{1}H NMR (300 MHz, CD_3OD): δ = 8.0 (s, 1 H, =CH), 5.41 (t, 1 H, $J_{2,3} = J_{3,4}$ = 9.8 Hz, H-3), 5.00 (t, 1 H, $J_{4,5}$ = 9.8 Hz, H-4), 4.92 (d, 1 H, $J_{1,2}$ = 3.6 Hz, H-1), 4.85 (dd, 1 H, H-2), 4.59 (t, 2 H, $J_{H,H}$ = 6.6 Hz, CH_2N), 3.92 (m, 1 H, H-5), 3.42 (bt, 2 H, $J_{H,H}$ = 6.0 Hz, C*H*$_2$NHCS), 3.42 (s, 3 H, OMe), 3.18 (bt, 2 H, $J_{H,H}$ = 6.0 Hz, C*H*$_2$$NH_2$), 3.13 (m, 2 H, CH_2S), 2.63 (m, 2 H, H-6a, H-6b), 2.42-2.12 (m, 6 H, H-2_{Hex}), 1.30 (m, 6 H, H-3_{Hex}), 1.30 (m, 12 H, H-4_{Hex}, H-5_{Hex}), 0.90 (m, 18 H, H-6_{Hex}).

^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 174.4, 174.0 (CO ester), 145.5 (C4 triazole), 124.8 (C5 triazole), 98.0 (C-1), 72.4 (C-4), 72.1 (C-2), 71.3 (C-5), 71.2 (C-3), 56.0 (OMe), 51.2 (CH_2N triazole), 42.5 (CH_2*C*H_2NHCS), 41.1 (CH_2*C*H_2NH_2), 35.0 (C-2_{Hex}), 34.2 (CH_2S), 34.0 (C-6), 32.3 (C-4_{Hex}), 25.6 (C-3_{Hex}), 23.4 (C-5_{Hex}), 14.2 (C-6_{Hex}).

ESIMS: *m/z* = 731.3 $[M]^+$. Anal. Calcd for $C_{33}H_{59}ClN_6O_8S_2$: C, 51.65; H, 7.75; N, 10.95; S, 8.36. Found: C, 51.45; H, 7.57; N, 10.68; S, 8.06.

Dendritic glucopyranoside derivative (65). To a solution of **62** (141 mg, 0.18 mmol) and Et_3N (75 μL, 0.54 mmol), *tert*-butyl *N*-(2-isothiocyanoethyl)carbamate **168** (109 mg, 0.54 mmol) was added, and the mixture was stirred at rt, under Ar atmosphere, for 48 h. The mixture was washed with HCl 0.1 N (2 x 10 mL) and the organic phase was dried ($MgSO_4$), filtered and concentrated. The residue was purified by column chromatography (3:1 → 1:0 EtOAc-cyclohexane → 45:5:3 EtOAc-EtOH-H_2O). Yield: 131 mg (65%). $[\alpha]_D$ = + 41.91 (*c* 1.0, DCM); UV (DCM): λ_{max} = 248 nm (ε_{mM} 20.5); IR: ν_{max} = 2959, 1747, 1701cm^{-1}.

^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.61 (s, 1 H, =CH), 7.15, 6.93 (bs, 2 H, NHCS), 5.46 (t, 1 H, $J_{2,3} = J_{3,4}$ = 9.7 Hz, H-3), 5.36 (s, 2 H, NHBoc), 4.98 (t, 1 H, $J_{4,5}$ = 9.7 Hz, H-4), 4.92 (d, 1 H, $J_{1,2}$ = 3.5 Hz, H-1), 4.83 (dd, 1 H, H-2), 4.50 (t, 4 H, $^3J_{H,H}$ = 6.7 Hz, SCH_2C*H*$_2$N), 3.94 (m, 1 H, H-5), 3.80 (s, 2 H, CH_2 triazole), 3.62 (bs, 4 H,

NHCSC*H*$_2$CH$_2$NHBoc), 3.54 (bs, 4 H, NCH$_2$C*H*$_2$NHCS), 3.41 (s, 3 H, OMe), 3.31 (m, 4 H, C*H*$_2$NHBoc), 3.12 (m, 2 H, CH$_2$S), 2.73 (m, 4 H, NC*H*$_2$CH$_2$NHCS), 2.60 (m, 2 H, H-6a, H-6b), 2.38-2.31 (m, 6 H, H-2$_{Hex}$), 1.55 (m, 6 H, H-3$_{Hex}$), 1.42 (s, 18 H, CMe$_3$), 1.26 (m, 12 H, H-4$_{Hex}$, H-5$_{Hex}$), 0.87 (m, 9 H, H-6$_{Hex}$).

^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 182.5 (CS), 173.0, 172.6, 172.5 (CO ester), 157.0 (CO carbamate), 144.5 (C4 triazole), 123.1 (C5 triazole), 96.7 (C-1), 79.8 (*C*Me$_3$), 71.1 (C-4), 70.8 (C-2), 69.9 (C-5), 69.4 (C-3), 55.6 (OMe), 52.7 (NC*H*$_2$CH$_2$NHCS), 50.0 (SCH$_2$*C*H$_2$N), 48.3 (CH$_2$-triazole), 45.1 (*C*H$_2$CH$_2$NHBoc), 42.1 (NCH$_2$*C*H$_2$NHCS), 40.2 (*C*H$_2$NHBoc), 34.0 (C-2$_{Hex}$), 33.5 (C-6), 33.4 (C*H*$_2$S), 31.1 (C-4$_{Hex}$), 28.4 (CMe$_3$), 24.5 (C-3$_{Hex}$), 22.2 (C-5$_{Hex}$), 13.8 (C-6$_{Hex}$).

ESIMS: *m/z* = 1181.5 $[M + Cu]^+$. Anal. Calcd for $C_{50}H_{90}N_{10}O_{12}S_3$: C, 53.64; H, 8.10; N, 12.51; S, 8.59. Found: C, 53.68; H, 8.15; N, 12.45; S, 8.41

Dendritic glucopyranoside derivative (66). Treatment of compound **65** (121 mg, 0.107 mmol) with 1:1 TFA:DCM (1 mL) as described in general methods yielded quantitatively **66**. Yield: 108 mg. $[\alpha]_D$ = + 42.71 (*c* 1.0, MeOH); UV (MeOH): λ_{max} = 243 nm (ε_{mM} 18.7); IR: ν_{max} = 2955, 1747, 1556, 721 cm^{-1}.

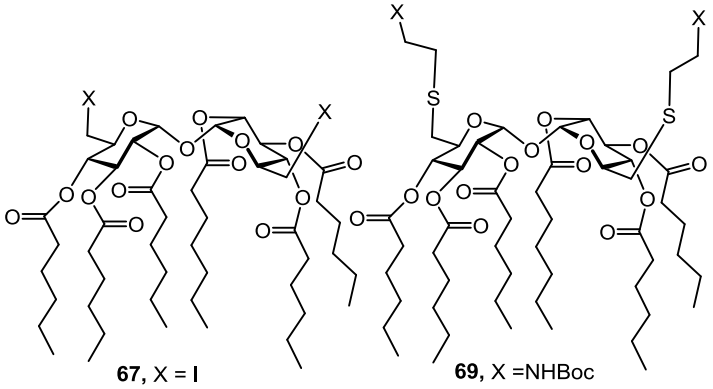
^{1}H NMR (300 MHz, CD_3OD): δ = 8.42 (s, 1 H, =CH), 5.41 (t, 1 H, $J_{2,3} = J_{3,4}$ = 9.5 Hz, H-3), 5.00 (t, 1 H, $J_{4,5}$ = 9.5 Hz, H-4), 4.94 (d, 1 H, $J_{1,2}$ = 3.6 Hz, H-1), 4.86 (dd, 1 H, H-2), 4.76 (s, 2 H, CH$_2$ triazole), 4.68 (bt, 2 H, $J_{H,H}$ = 6.5 Hz, SCH$_2$C*H*$_2$N), 4.06 (bt, 4 H, C*H*$_2$CH$_2$NH$_2$), 3.94 (ddd, 1 H, $J_{5,6a}$= 3.0 Hz, $J_{5,6b}$= 7.0 Hz, H-5), 3.87 (bt, 4 H, $J_{H,H}$ = 5.7 Hz, NCH$_2$C*H*$_2$NHCS), 3.44 (s, 3 H, OMe), 3.20 (m, 6 H, NC*H*$_2$CH$_2$NHCS, CH$_2$S), 2.71 (dd, 1 H, $J_{6a,6b}$ = 14.5 Hz, H-6a), 2.61 (dd, 1 H, H-6b), 2.43-2.19 (m, 6 H, H-2$_{Hex}$), 1.56 (m, 6 H, H-3$_{Hex}$), 1.30 (m, 12 H, H-4$_{Hex}$, H-5$_{Hex}$), 0.91 (m, 9 H, H-6$_{Hex}$).

^{13}C NMR (75.5 MHz, CD_3OD): δ = 185.9 (CS), 174.3,174.1, 174.0 (CO), 137.1 (C4 triazole), 129.2 (C5 triazole), 98.0 (C-1), 72.3 (C-4), 72.1 (C-2), 71.2 (C-5, C-3), 56.0 (OMe), 54.7 (NC*H*$_2$CH$_2$NH$_2$), 52.1 (SCH$_2$*C*H$_2$N), 42.6 (H$_2$NCH$_2$C*H*$_2$NHCS), 40.9

(NCH_2CH_2NHCS), 40.2 (CH_2NH_2), 35.0 (C-2_{Hex}), 34.2 (C-6), 34.0 (CH_2S), 32.3 (C-4_{Hex}), 25.6 (C-3_{Hex}) 23.4 (C-5_{Hex}), 14.2 (C-6_{Hex}).

ESIMS: *m/z* = 1793 $[M + H]^+$, 981.4 $[M + Cu]^+$. Anal. Calcd for $C_{40}H_{76}Cl_2N_{10}O_8S_3$: C, 48.42; H, 7.15; N, 14.12; S, 9.70. Found: C, 48.19; H, 7.56; N, 13.87; S, 9.51.

Preparation of cationic amphiphilic derivatives of trehalose.



67, X = I
68, X = N_3

69, X =NHBoc
70, X = NH_2·HCl
71, X = N_3

6,6'-Dideoxy-2,3,4,2',3',4'-hexa-*O*-hexanoyl-6,6'-diiodo-α,α'-trehalose (67). To a solution of 6,6'-dideoxy-6,6'-diiodo-α,α'-trehalose **176** (5.78 g, 10.3 mmol) and DMAP (6.37 g, 52.2 mmol) in dry DMF (80 mL), hexanoic anhydride (16 mL, 69.6 mmol) was dropwise added, under Ar atmosphere, at 0º C and the reaction mixture was stirred at rt for 6 h. Then, MeOH (60 mL) was added and the mixture was stirred ad rt for 2 h. The product was poured onto H_2O-ice mixture (100 mL) and DCM (50 mL) was added. The organic phase was then washed with 2N H_2SO_4 (2 x 50 mL), H_2O (2 x 50 mL) and saturated aqueous $NaHCO_3$ (2 x 50 mL), dried ($MgSO_4$), filtered, concentrated and purified by column chromatography (1:12 EtOAc-petroleum ether). Yield: 1.2 g (76%); R_f = 0.35 (1:6 EtOAc-petroleum ether).

^{1}H NMR (300 MHz, $CDCl_3$): δ = 5.44 (t, 2 H, $J_{2,3} = J_{3,4}$ = 9.5 Hz, H-3), 5.35 (d, 2 H, $J_{1,2}$ = 3.7 Hz, H-1), 5.1 (dd, 2 H, H-2), 4.84 (t, 2 H, $J_{4,5}$ = 9.5 Hz, H-4), 3.82 (ddd, 2 H,

$J_{5,6a}$ = 2.5 Hz, H-5), 3.11 (dd, 2 H, $J_{6a,6b}$ = 11.0 Hz, H-6a), 2.97 (dd, 2 H, H-6b), 2.22 (m, 12 H, H-2_{Hex}), 1.58 (m, 12 H, H-3_{Hex}), 1.17 (m, 24 H, H-4_{Hex}, H-5_{Hex}), 0.84 (m, 18 H, H-6_{Hex}).

^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 172.6, 172.5, 172.4 (CO), 91.5 (C-1), 72.0 (C-4), 70.0 (C-5), 69.3 (C-3), 69.2 (C-2), 34 (C-2_{Hex}), 31.2 (C-4_{Hex}), 24.4 (C-3_{Hex}), 22.2 (C-5_{Hex}), 14.1 (C-6_{Hex}), 2.6 (C-6_{Hex}).

ESIMS: *m/z* = 1173.4 $[M + Na]^+$. Anal. Calcd for $C_{47}H_{78}I_2O_{15}$: C, 49.65; H, 6.92. Found: C, 50.12; H, 7.01.

6,6'-Diazido-6,6'-dideoxy-2,3,4,2',3',4'-hexa-*O*-hexanoyl) α,α'-trehalose (68). To a solution of **67** (1.58 g, 1.37 mmol) in dry DMF (8 mL), NaN_3 (250 mg, 3.6 mmol) was added. The reaction mixture was stirred overnight at 40 ºC, under Ar atmosphere. The mixture was poured into ice-water (20 mL), and the product was extracted with DCM (4 x 20 mL). The organic phase was dried ($MgSO_4$), filtered and concentrated. The residue was purified by column chromatography (1:12 EtOAc-cyclohexane). Yield: 913 mg (72%). R_f = 0.53 (1:6 EtOAc-cyclohexane); $[\alpha]_D$ = +108.7 (*c* 1.0, DCM); IR: ν_{max} = 2958, 2104, 1750, 735 cm^{-1}.

^{1}H NMR (300 MHz, CD_3OD): δ = 5.52 (t, 2 H, $J_{2,3}$ = $J_{3,4}$ = 9.8 Hz, H-3), 5.41 (d, 2 H, $J_{1,2}$ = 4.0 Hz, H-1), 5.13 (dd, 2 H, H-2), 5.09 (t, 2 H, $J_{4,5}$ = 9.8 Hz, H-4), 4.08 (ddd, 2 H, $J_{5,6a}$ = 7.0 Hz, $J_{5,6b}$ = 2.6 Hz, H-5), 3.44 (dd, 2 H, $J_{6a,6b}$ = 13.0 Hz, H-6a), 2.97 (dd, 2 H, H-6b), 2.33 (m, 12 H, H-2_{Hex}), 1.58 (m, 12 H, H-3_{Hex}), 1.33 (m, 24 H, H-4_{Hex}, H-5_{Hex}), 0.91 (m, 18 H, H-6_{Hex}).

^{13}C NMR (75.5 MHz, CD_3OD): δ = 175.2-174.2 (CO), 93.7 (C-1), 71.5 (C-3), 71.3 (C-2), 71.6 (C-5), 71 (C-4), 52.2 (C-6), 34.9 (C-2_{Hex}), 33.2 (C-4_{Hex}), 25.8 (C-3_{Hex}), 23.6 (C-5_{Hex}), 14.8 (C-6_{Hex}).

ESIMS: *m/z* = 1003.5 $[M + Na]^+$. Anal. Calcd for $C_{25}H_{43}N_8O_{15}$: C, 58.76; H, 8.22; N, 8.57. Found: C, 58.84; H, 8.32; N, 8.60.

2,3,4,2',3',4'-Hexa-*O*-hexanoyl-6,6'-bis[2-*tert*-butoxycarbonyl-aminoethylthio]-α,α'-trehalose (69). To a solution of **67** (195 mg, 0.169 mmol) in dry DMF (1.5 mL), Cs_2CO_3 (154.6, 0.474 mmol) and *tert*-butyl *N*-(2-mercaptoethyl)carbamate (80 μL, 0.474 mmol) were added, under Ar atmosphere, and the reaction mixture was stirred at 60º C for 24 h . The reaction mixture was concentrated and the crude product was dissolved in DCM (10 mL) and washed with water (2 x 20 mL). The organic phase was dried ($MgSO_4$), filtered and concentrated. The residue was purified by column chromatography (1:3 EtOAc-cyclohexane). Yield: 124 mg (58%). R_f = 0.52 (1:2 EtOAc-cyclohexane); $[\alpha]_D$ = +84.5 (*c* 1, DCM); IR: ν_{max} = 2959, 1749, 1709 cm^{-1}.

^{1}H NMR (300 MHz, $CDCl_3$): δ = 5.43 (t, 2 H, $J_{3,2} = J_{3,4}$= 9.5 Hz, H-3), 5.25 (d, 2 H, $J_{1,2}$= 4.1 Hz, H-1), 5.00 (dd, 2 H, H-2), 4.94 (t, 2 H, H-4), 3.88 (m, 2 H, H-5), 3.2 (s, 4 H, CH_2N), 2.61 (m, 4 H, CH_2S), 2.52 (m, 4 H, H-6a, H-6b), 2.23 (m, 12 H, H-2_{Hex}), 1.50 (m, 12 H, H-3_{Hex}), 1.41 (s, 18 H, CMe_3), 1.23 (m, 24 H, H-4_{Hex}, H-5_{Hex}), 0.83 (t, 18 H, $J_{H,H}$ = 6.0 Hz, H-6_{Hex}).

^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 172.6, 172.5, 172.4 (C-1_{Hex}), 155.7 (CO carbamate), 91.4 (C-1), 71.2 (C-4), 71.1 (C-5), 69.6 (C-2, C-3), 39.7 (CH_2N), 34.1, 34.0 (C-2_{Hex}), 33.8 (CH_2S), 24.4 (C-3_{Hex}), 28.4 (CMe_3), 22.3 (C-4_{Hex}, C-5_{Hex}), 13.8 (C-6_{Hex}).

ESIMS: *m/z* = 1271.8 $[M + Na]^+$. Anal. Calcd for $C_{62}H_{108}N_2O_{19}S_2$: C, 59.59; H, 8.71; N, 2.24; S, 5.13. Found: C, 59.67; H, 8.69; N, 2.32; S, 4.89.

2,3,4,2',3',4'-Hexa-*O*-hexanoyl-6,6'-bis[2-*tert*-aminoethylthio]-α,α'-trehalose dihydrochloride (70). Treatment of **69** (77 mg, 0.04 mmol) with 1:1 TFA:DCM (2 mL) as indicated in general methods yielded quantitatively **70**. Yield: 71 mg. $[\alpha]_D$ = + 66.7 (*c* 0.91, EtOAc); IR: ν_{max} = 2957, 1740, 1686 cm^{-1}.

^{1}H NMR (300 MHz, DMSO-d_6): δ = 5.35 (t, $J_{2,3}$ = 9.7 Hz, 2 H, H-3), 5.29 (d, 2 H, $J_{1,2}$ = 4.0 Hz, H-1), 5.08 (m, 4 H, H-2, H-3), 3.95 (m, 2 H, H-5), 2.95 (bs, 4 H, CH_2N), 2.69 (t,

4 H, $J_{H,H}$ = 7.0 Hz, CH_2S), 2.67 (bd, 4 H, H-6a, H-6b), 2.27 (m, 12 H, H-2_{Hex}), 1.49 (m, 12 H, H-3_{Hex}), 1.24 (m, 24 H, H-4_{Hex}, H-5_{Hex}), 0.85 (t, 18 H, $J_{H,H}$ = 6.0 Hz, H-6_{Hex}).

^{13}C NMR (75.5 MHz, DMSO-d_6): δ = 171.8, 171.7 (CO), 90.9 (C-1), 70.9 (C-5), 70.0, 69.2, (C-2, C-4), 69.4 (C-3), 39.2 (CH_2N), 31.5 (C-6), 33.9 (C-2_{Hex}), 30.3 (CH_2S), 23.9 (C-3_{Hex}), 30.6 (C-4_{Hex}), 21.8 (C-5_{Hex}), 13.3 (C-6_{Hex}).

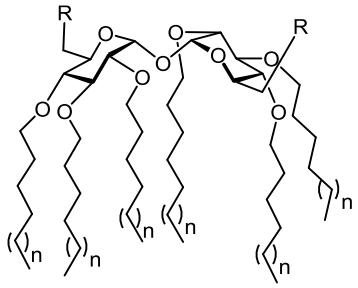
ESIMS: *m/z* = 1049.6 $[M]^+$. Anal. Calcd for $C_{52}H_{94}Cl_2N_2O_{15}S_2$: C, 55.65; H, 8.44; N, 2.50; S, 5.71. Found: C, 55.29; H, 8.18; N, 2.14; S, 5.33.

6,6'-Di-(2-azidoehylthio)-2,3,4,2',3',4'-hexa-*O*-hexanoyl)-α,α'-trehalose (71). To a solution of **70** (956 mg, 0.85 mmol), $NaHCO_3$ (286 mg, 3.41 mmol) and $CuSO_4 \cdot 5\ H_2O$ (8.5 mg, 0.034 mmol) in H_2O (6.25 mL), triflic azide solution (10.6 mL) was added, followed by addition of MeOH (42 mL) to yield a homogeneous system. Subsequently, the blue mixture was stirred vigorously at rt for 24 h. The mixture was concentrated at reduced pressure at rt. The residue was purified by column chromatography (1:8 → 1:4 EtOAc-cyclohexane). Yield: 550 mg (59%). R_f = 0.51 (1:2 EtOAc-cyclohexane); $[\alpha]_D$ = + 109.9 (*c* 1.0, DCM); IR: ν_{max} = 2957, 2102, 1749 cm^{-1}.

^{1}H NMR (300 MHz, $CDCl_3$): δ = 5.49 (t, 2 H, $J_{2,3} = J_{3,4}$ = 9.6 Hz, H-3), 5.31 (d, 2 H, $J_{1,2}$ = 3.7 Hz, H-1), 5.08 (dd, 2 H, H-2), 5.02 (t, 2 H, $J_{4,5}$ = 9.6 Hz H-4), 3.96 (m, 2 H, H-5), 3.42 (t, 4 H, $J_{H,H}$ = 7.0 Hz, CH_2N), 2.74 (m, 4 H, CH_2S), 2.63 (m, 4 H, H-6a, H-6b), 2.42-2.10 (m, 12 H, H-2_{Hex}), 1.58 (m, 12 H, H-3_{Hex}), 1.29 (m, 24 H, H-4_{Hex}, H-5_{Hex}), 0.89 (t, 18 H, $J_{H,H}$ = 6.0 Hz, H-6_{Hex}).

^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 172.8, 171.7 (CO ester), 91.6 (C-1), 71.3 (C-5), 71.1 (C-2), 69.6 (C-4, C-3), 51.1 (C-6), 33.2 (CH_2N), 32.6 (CH_2S), 34.1 (C-2_{Hex}), 31.2 (C-4_{Hex}), 24.5 (C-3_{Hex}), 22.2 (C-5_{Hex}), 13.9 (C-6_{Hex}).

ESIMS: *m/z* = 1023.7 $[M]^+$. Anal. Calcd for $C_{68}H_{114}N_8O_{19}S_2$: C, 57.85; H, 8.14; N, 7.94; S, 4.54. Found: C, 56.79; H, 7.96; N, 7.54; S, 5.68



72, n=1, R = OTr
73, n=9, R = OTr
74, n=1, R = OH
75, n=9, R = OH
76, n=1, R = I
77, n=9, R = I
78, n=1, R = N_3
79, n=9, R = N_3
80, n=1, R = $NH_2 \cdot HCl$
81, n=9, R = $NH_2 \cdot HCl$

2,3,4,2',3',4'-Hexa-*O*-hexyl-6,6'-di-*O*-trityl-α,α'-trehalose (72). To a solution of **32** (1.00 g, 1.21 mmol) in dry DMF (11 mL), NaH (871 mg, 21.78 mmol) was added and the mixture was stirred at 0 ºC for 10 min. 1-Bromohexane (3.06 mL, 21.78 mmol) was added dropwise under Ar atmosphere and the mixture was stirred overnight at rt. The reaction was quenched with MeOH (5 mL) and stirred for 10 min. The solvents were removed and the resulting residue was suspended in DCM (50 mL), washed with H_2O (3 x 15 mL) and the organic layer was separated, dried ($MgSO_4$), filtered, concentrated, and purified by column chromatography (1:8→1:6 EtOAc-cyclohexane). Yield: 1.50 g (92%). R_f = 0.74 (1:5 EtOAc-cyclohexane); $[\alpha]_D$ = +70.3 (*c* 1.0, DCM); IR: ν_{max} = 2923, 2855 cm^{-1}.

^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.53-7.23 (m, 30 H, Ph), 5.34 (d, 2 H, $J_{1,2}$ = 3.7 Hz, H-1), 4.03 (bd, 2 H, $J_{4,5}$ = 9.3 Hz, H-5), 3.80 (m, 2 H, OCH_2), 3.78-3.41 (m, 8 H, OCH_2), 3.57 (t, 2 H, $J_{2,3} = J_{3,4}$ = 9.3 Hz, H-3), 3.51-3.41 (m, 4 H, H-4, H-6a), 3.39 (dd, 2 H, H-2), 3.24 (m, 2 H, OCH_2), 3.13 (dd, 2 H, $J_{6a,6b}$ = 10.0 Hz, $J_{5,6b}$ = 3.3 Hz, H-6b), 1.65-1.56 (m, 12 H, CH_2), 1.38-1.04 (m, 36 H, CH_2), 0.93-0.82 (m, 18 H, CH_3).

^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 144.0, 128.8, 127.6, 126.8 (Ph), 93.6 (C-1), 86.1 (*C*Ph_3), 81.5 (C-3), 80.6 (C-2), 78.1 (C-4), 73.7, 73.0, 71.3 (OCH_2), 70.0 (C-5), 62.0 (C-6), 31.8-22.5 (CH_2), 14.1 (CH_3).

ESIMS: *m/z* = 1353.8 $[M + Na]^+$. Anal. Calcd for $C_{86}H_{122}O_{11}$: C, 77.55; H, 9.23. Found: C, 77.67; H, 9.31.

2,3,4,2',3',4-Hexa-*O*-tetradecyl-6,6'-di-*O*-trityl-α,α'-trehalose (73). To a solution of **32** (1.00 g, 1.21 mmol) in dry DMF (11 mL), NaH (0.87 g, 21.78 mmol) was added and the mixture was stirred at 0 ºC for 10 min. 1-Bromotetradecane (6.68 mL, 21.78 mmol) was added dropwise, under Ar atmosphere, and the mixture was stirred overnight at 60 ºC. The reaction was quenched with MeOH (5 mL) and stirred for 10 min. Solvents were removed and the resulting residue was suspended in DCM (50 mL). The suspension was washed with H_2O (3 x 15 mL) and the organic layer was dried ($MgSO_4$), filtered, concentrated and purified by column chromatography (1:50 → 1:30 EtOAc-cyclohexane). Yield: 1.50 g (77%). R_f = 0.77 (1:15 EtOAc-cyclohexane); $[\alpha]_D$ = +50.2 (*c* 1.0, DCM).

^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.52-7.22 (m, 30 H, Ph), 5.33 (d, 2 H, $J_{1,2}$ = 3.6 Hz, H-1), 4.03 (bd, 2 H, $J_{4,5}$ = 9.4 Hz, H-5), 3.79 (m, 2 H, OCH_2), 3.71-3.52 (m, 8 H, OCH_2), 3.55(t, 2 H, $J_{2,3}$ = $J_{3,4}$ = 9.4 Hz, H-3),3.44 (t, 2 H, H-4), 3.46-3.35 (m, 6 H, OCH_2, H-6a, H-2), 3.22 (m, 2 H, OCH_2), 3.12 (dd, 2 H, $J_{6a,6b}$ = 10.0 Hz, $J_{5,6b}$ = 3.1 Hz, H-6b), 1.76-156 (m, 12 H, CH_2), 1.28 (m, 132 H, CH_2), 0.91 (t, 18 H, $J_{H,H}$ = 6.3 Hz, CH_3).

^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 144.1, 128.8, 127.6, 126.8 (Ph), 93.7 (C-1), 86.2 (Ph_3C), 81.4 (C-3), 80.6 (C-2), 78.2 (C-4), 73.7, 73.0, 71.3 (OCH_2), 70.5(C-5), 62.1 (C-6), 31.9- 22.7 (CH_2), 14.1 (CH_3).

ESIMS: *m/z* = 2027.4 $[M + Na]^+$. Anal. Calcd for $C_{134}H_{218}O_{11}$: C, 80.26; H, 10.96. Found: C, 80.35; H, 11.05.

2,3,4,2',3',4-Hexa-*O*-hexyl-α,α'-trehalose (74). To a solution of **72** (0.68 g, 0.52 mmol) in 1:1 DCM-MeOH (25 mL), PTSA monohydrate (0.08 g, 0.42 mmol) was added and the solution was stirred at rt for 4 h. The mixture was diluted with DCM, washed with saturated aqueous $NaHCO_3$, dried ($MgSO_4$), filtered and concentrated. The resulting residue was purified by column chromatography (1:8 → 1:2 EtOAc-cyclohexane). Yield: 0.21 g (48%); R_f = 0.25 (1:2 EtOAc-cyclohexane); $[\alpha]_D$ = +103.0 (*c* 1.0, DCM).

^{1}H NMR (300 MHz, $CDCl_3$): δ = 5.06 (d, 2 H, $J_{1,2}$ = 3.7 Hz, H-1), 3.90 (dt, 2 H, $J_{4,5}$ = 9.2 Hz, $J_{5,6a}$ = $J_{5,6b}$ = 2.9 Hz, H-5), 3.79 (m, 4 H, OCH_2), 3.73-3.63 (m, 8 H, H-6a, H-6b,

OCH_2), 3.60-3.43 (m, 4 H, OCH_2), 3.57 (t, 2 H, $J_{2,3}$= $J_{3,4}$= 9.2 Hz, H-3), 3.23 (t, 1 H, H-4), 3.18 (dd, 1 H, H-2), 2.03 (bs, 2 H, OH), 1.60-1.47 (m, 12 H, CH_2), 1.37-1.27 (m, 36 H, CH_2), 0.87 (m, 18 H, CH_3).

^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 93.6 (C-1), 81.0 (C-3), 80.4 (C-2), 77.9 (C-4), 73.6, 73.3, 71.5 (OCH_2), 71.4 (C-5), 61.8 (C-6), 31.8- 22.6 (CH_2), 14.0 (CH_3).

ESIMS: *m/z* = 869.7 $[M + Na]^+$, 885.7 $[M + K]^+$. Anal. Calcd for $C_{48}H_{94}O_{11}$: C, 68.05; H, 11.18. Found: C, 67.89; H, 11.04.

2,3,4,2',3',4-Hexa-*O*-tetradecyl-α,α'-trehalose (75). To a solution of **73** (1.49 g, 0.74 mmol) in 1:1 DCM-MeOH (36 mL), PTSA (0.11 g, 0.50 mmol) was added and the solution was stirred at rt for 4 h. The mixture was diluted with DCM, washed with saturated aqueous $NaHCO_3$, dried ($MgSO_4$), filtered, concentrated and purified by column chromatography (1:9 → 1:5 EtOAc-cyclohexane). Yield: 0.53 g (47%). R_f = 0.20 (1:5 EtOAc-cyclohexane); $[\alpha]_D$ = +58.4 (*c* 1.0, DCM).

^{1}H NMR (300 MHz, $CDCl_3$): δ = 5.06 (d, 2 H, $J_{1,2}$ = 3.7 Hz, H-1), 3.90 (dt, 2 H, $J_{4,5}$ = 9.2 Hz, $J_{5,6a}$ = $J_{5,6b}$ = 2.6 Hz, H-5), 3.84-3.64 (m, 4 H, OCH_2), 3.73-3.63 (m, 12 H, H-6a, H-6b, OCH_2), 3.58 (t, 2 H, $J_{2,3}$ = $J_{3,4}$ = 9.2 Hz, H-3), 3.24 (t, 2 H, H-4), 3.19 (dd, 2 H, H-2), 1.86 (bs, 2 H, OH), 1.61-1.49 (m, 12 H, CH_2), 1.26 (bs, 132 H, CH_2), 0.88 (t, 18 H, $J_{H,H}$ = 6.9 Hz, CH_3).

^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 93.7 (C-1), 81.1 (C-3), 80.5 (C-2), 78.0 (C-4), 73.6, 73.3, 71.6 (OCH_2), 71.1 (C-5), 61.9 (C-6), 31.9- 22.7 (CH_2), 14.1 (CH_3).

ESIMS: *m/z* = 1543.2 $[M + Na]^+$. Anal. Calcd for $C_{96}H_{190}O_{11}$: C, 75.83; H, 12.59. Found: C, 75.70; H, 12.41.

6,6'-Dideoxy-2,3,4,2',3',4-hexa-*O*-hexyl-6,6'-diiodo-α,α'-trehalose (76). To a solution of **74** (0.10 g, 0.12 mmol) in toluene (5 mL), TPP (0.11 g, 0.43 mmol) and imidazole (0.05 g, 0.81 mmol) were added and the mixture was stirred at rt until complete dissolution. Iodine (0.11 g, 0.40 mmol) was added in portions and the solution was

vigorously stirred at 70 ºC for 5 h. Saturated aqueous $NaHCO_3$ solution (10 mL) was added and the mixture was stirred for 5 min. Additional iodine was then added until the aqueous solution turned to a slightly brown color, then aqueous 10% $Na_2S_2O_3$ was added until complete decoloration of both organic and aqueous layer. The organic layer was then separated, dried ($MgSO_4$), filtered, concentrated and purified by column chromatography (1:15 EtOAc-cyclohexane). Yield: 0.12 g (94%). R_f = 0.75 (1:8 EtOAc-cyclohexane); $[\alpha]_D$ = +50.1 (*c* 1.0, DCM).

^{1}H NMR (300 MHz, $CDCl_3$): δ = 5.17 (d, 2 H, $J_{1,2}$ = 3.3 Hz, H-1), 3.83 (m, 4 H, OCH_2), 3.72-3.47 (m, 12 H, H-5, H-3, OCH_2), 3.39 (m, 4 H, H-6a, H-6b), 3.24 (dd, 2 H, Hz, $J_{2,3}$ = 9.1 Hz, H-2), 3.05 (t, 2 H, $J_{3,4}$ = $J_{4,5}$ = 9.1 Hz, H-4), 1.62-1.49 (m, 12 H, CH_2), 1.30 (m, 36 H, CH_2), 0.89 (m, 18 H, CH_3).

^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 92.9 (C-1), 81.9 (C-4), 80.8 (C-3), 80.2 (C-2), 73.5, 73.4, 71.9 (OCH_2), 69.1 (C-5), 31.8- 22.6 (CH_2), 14.0 (CH_3), 8.7 (C-6).

ESIMS: *m/z* = 1089.6 $[M + Na]^+$. Anal. Calcd for $C_{48}H_{92}I_2O_9$: C, 54.03; H, 8.69. Found: C, 53.88; H, 8.77.

6,6'-Dideoxy-2,3,4,2',3',4-hexa-*O*-tetradecyl-6,6'-diiodo-α,α'-trehalose (77). To a solution of **75** (0.44 g, 0.29 mmol) in toluene (13 mL), TPP (0.27 g, 1.02 mmol) and imidazole (0.07 g, 1.89 mmol) were added and the mixture was stirred at rt until complete dissolution. Iodine (0.26 g, 0.93 mmol) was added in portions and the solution was vigorously stirred at 70 ºC for 5 h. Saturated aqueous $NaHCO_3$ (20 mL) was added and the mixture was stirred for 5 min. Additional iodine was then added until the aqueous solution got slightly brown, and then an 10%aqueous $Na_2S_2O_3$ solution was added until complete decoloration of both organic and aqueous layer. The organic layer was then separated, dried ($MgSO_4$), filtered, concentrated, and purified by column chromatography (1:25 EtOAc-cyclohexane). Yield: 0.52 g (96%). R_f = 0.69 (1:20 EtOAc-cyclohexane); $[\alpha]_D$ = +47.2 (*c* 1.0, DCM).

^{1}H NMR (300 MHz, $CDCl_3$): δ = 5.17 (d, 2 H, $J_{1,2}$ = 3.5 Hz, H-1), 3.83 (m, 4 H, OCH_2), 3.70-3.48 (m, 12 H, H-5, H-3, OCH_2), 3.42 (dd, 2 H, $J_{6a,6b}$ = 10.7 Hz, $J_{5,6a}$ = 2.9 Hz, H-6a), 3.36 (dd, 2 H, $J_{5,6a}$ = 5.2 Hz, H-6b), 3.24 (dd, 2 H, Hz, $J_{2,3}$ = 9.4 Hz, H-2), 3.05 (t, 2 H, $J_{3,4}$ = $J_{4,5}$ = 9.4 Hz, H-4), 1.59-1.51 (m, 12 H, CH_2), 1.26 (bs, 132 H, CH_2), 0.88 (t, 18 H, $J_{H,H}$ = 6.9 Hz, CH_3).

^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 92.9 (C-1), 81.9 (C-4), 80.8 (C-3), 80.3 (C-2), 73.6, 73.5, 71.9 (OCH_2), 69.1 (C-5), 31.9- 22.7 (CH_2), 14.1 (CH_3), 8.7 (C-6).

ESIMS: *m/z* = 1763.0 $[M + Na]^+$. Anal. Calcd for $C_{96}H_{188}I_2O_9$: C, 66.25; H, 10.89. Found: C, 66.09; H, 10.74.

6,6'-Diazido-6,6'-dideoxy-2,3,4,2',3',4'-hexa-*O*-hexyl-α,α'-trehalose (78). Compound **78** was obtained by treatment of **76** (0.450 g, 0.42 mmol) with NaN_3 (82 mg, 1.26 mmol) in DMF (2.5 mL). Column chromatography of the residue (1:10 → 1:8 EtOAc-cyclohexane) afforded **78**. Yield: 362 mg (96%). R_f = 0.57 (1:10 EtOAc-cyclohexane); $[\alpha]_D$ = +97 (*c*1.0 in DCM).

^{1}H NMR (300 MHz, $CDCl_3$): δ = 5.11 (d, 2 H, $J_{1,2}$ = 3.5 Hz, H-1), 4.04 (ddd, 2 H, $J_{4,5}$ = 9.5 Hz, $J_{5,6a}$ = 4.2 Hz, $J_{5,6b}$ = 2.9 Hz, H-5), 3.81 (m, 4 H, OCH_2), 3.64-3.34 (m, 14 H, OCH_2, H-3, H-6a, H-6b), 3.25 (dd, 2 H, $J_{2,3}$ = 9.5 Hz, H-2), 3.16 (t, 2 H, $J_{3,4}$ = 9.5 Hz H-4), 1.62-1.48 (m, 12 H, CH_2), 1.34-1.24 (bs, 36 H, CH_2), 0.88 (m, 18 H, CH_3).

^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 93.5 (C-1), 81.6, 80.3 (C-3, C-2), 78.7 (C-4), 73.5, 73.3, 71.9 (OCH_2), 70.6 (C-5), 51.5 (C-6), 31.8, 31.7, 30.6, 30.3, 30.2, 25.9, 25.8, 22.6 (CH_2), 14.0 (CH_3).

ESIMS: *m/z* = 919.8 $[M + Na]^+$. Anal. Calcd for $C_{48}H_{92}N_6O_9$: calcd. C, 64.25; H, 10.33; N, 9.37. Found: C, 64.31; H, 10.27; N, 917.

6,6'-Diazido-6,6'-dideoxy-2,3,4,2',3',4'-hexa-*O*-tetradecyl-α,α'-trehalose (79). Compound **79** was obtained by treatment of **77** (0.248 g, 0.142 mmol) with NaN_3 (56 mg,

0.855 mmol) in DMF (5 mL). Column chromatography (1:40 EtOAc-cyclohexane) afforded **79**. Yield: 193mg (87%). R_f = 0.26 (1:40 EtOAc-cyclohexane); $[\alpha]_D$ = +68 (*c* 1.0 in DCM); IR: ν_{max} = 2922, 2852, 2099, 1095 cm^{-1}.

^1H NMR (300 MHz, $CDCl_3$): δ = 5.11 (d, 2 H, $J_{1,2}$ = 3.7 Hz, H-1), 4.04 (ddd, 2 H, $J_{4,5}$ = 9.7 Hz, $J_{5,6a}$ = 4.4 Hz, $J_{5,6b}$ = 2.9 Hz, H-5), 3.81 (m, 4 H, OCH_2), 3.70-3.47 (m,10 H, OCH_2, H-3), 3.40 (m, 4 H, H-6a, H-6b), 3.25 (dd, 2 H, $J_{2,3}$ = 9.7 Hz, H-2), 3.16 (t, 2 H, $J_{3,4}$ = 9.7 Hz, H-4), 1.60-1.48 (m, 12 H, CH_2), 1.26 (bs, 132 H, CH_2), 0.88 (t, 18 H, $J_{H,H}$ = 6.7 Hz, CH_3).

^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 93.5 (C-1), 81.0 (C-3), 80.3 (C-2), 78.7 (C-4), 73.6, 73.4, 71.7 (OCH_2), 70.5 (C-5), 51.4 (C-6), 31.9, 30.7, 30.4, 30.2, 29.7, 29.6, 29,4, 26.3, 26.2, 22.7 (CH_2), 14.1 (CH_3).

ESIMS: *m/z* = 1592.4 $[M + Na]^+$. Anal. Calcd for $C_{96}H_{188}N_6O_9$: C, 73.42; H, 12.07; N, 5.35. Found: C, 74.61; H, 12.12; N, 5.27.

6,6'-Diamino-6,6'-dideoxy-2,3,4,2',3',4'-hexa-*O*-hexyl-α,α'-trehalose dihydrochloride (80). To a solution of **78** (250 mg, 0.278 mmol) in THF (22.5 mL), TPP (293 mg, 0.39 mmol) was added and the mixture was stirred at rt for 15 min. Then NH_4OH (2.5 mL) was added and the solution was stirred overnight at 50 ºC. The mixture was concentrated, the resulting residue purified by column chromatography (EtOAc → 45:5:3 AcOEt-EtOH-H_2O → 5:1 DCM-MeOH) and freeze-drying affording **80**. Yield: 137 mg (58%). R_f = 0.40 (5:1 DCM-MeOH); $[\alpha]_D$ = +95 (*c* 1.1 in DCM); IR: ν_{max} = 2928, 2855, 1099, 993 cm^{-1}.

^1H NMR (300 MHz, $CDCl_3$): δ = 5.06 (d, 2 H, $J_{1,2}$ = 3.5 Hz, H-1), 3.79 (m, 6 H, OCH_2, H-5), 3.71-3.46 (m, 8 H, OCH_2), 3.59 (t, 2 H, $J_{2,3}$ = $J_{3,4}$ = 9.5 Hz, H-3), 3.19 (dd, 2 H, H-2), 3.09 (t, 2 H, $J_{4,5}$ = 9.5 Hz, H-4), 2.93 (dd, 2 H, $J_{5,6a}$ = 2.4 Hz, $J_{6a,6b}$ = 13.5 Hz, H-6a), 2.77 (dd, 2 H, $J_{5,6b}$ = 5.5 Hz, H-6b), 1.59-1.49 (m, 12 H, CH_2), 1.34-1.27 (m, 36 H, CH_2), 0.90-0.84 (m,18 H, CH_3).

^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 93.0 (C-1), 81.2 (C-3), 80.5 (C-2), 79.2 (C-4), 73.4, 73.2 (OCH_2), 71.8 (C-5), 71.6 (OCH_2), 42.7 (C-6), 31.8, 30.4, 30.6, 30.2, 25.9, 25.8, 22.6 (CH_2), 14.0 (CH_3).

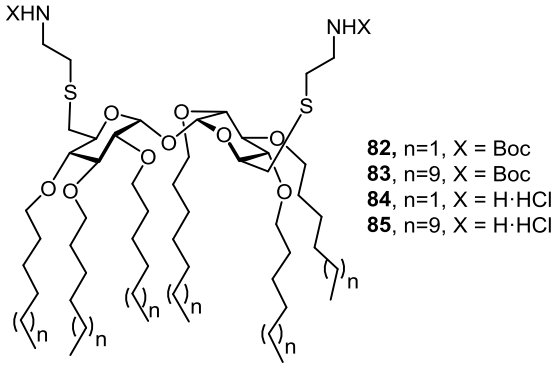
ESIMS: *m/z* = 845.6 $[M + H]^+$. Anal. Calcd for $C_{48}H_{98}Cl_2N_2O_9$: C, 62.79; H, 10.76; N, 3.05. Found: C, 62.43; H, 10.41; N, 2.87.

6,6'-Diamino-6,6'-dideoxy-2,3,4,2',3',4'-hexa-*O*-tetradecyl-α,α'-trehalose dihydrochloride (81). To a solution of **79** (447 mg, 0.285 mmol) in THF (23 mL), TPP (105 mg, 0.40 mmol) was added and the mixture was stirred at rt for 15 min. Then NH_4OH (2.6 mL) was added and the solution was stirred overnight at 50 ºC. The mixture was concentrated, the resulting residue purified by column chromatography (EtOAc → 45:5:3 AcOEt-EtOH-H_2O → 5:1 DCM-MeOH) and freeze-drying affording **81**. Yield: 137 mg (59%). R_f = 0.47 (9:1 DCM-MeOH); $[\alpha]_D$ = +24 (*c* 1.0 in DCM); IR: ν_{max} = 2918, 2850, 1096, 1007 cm^{-1}.

^{1}H NMR (300 MHz, 10:1 $CDCl_3$-CD_3OD): δ = 4.97 (d, 2 H, $J_{1,2}$ = 3.5 Hz, H-1), 3.70 (m, 6 H, OCH_2, H-5), 3.61-3.37 (m, 8 H, OCH_2), 3.47 (t, 2 H, $J_{2,3}$ = $J_{3,4}$ = 9.4 Hz, H-3), 3.12 (dd, 2 H, H-2), 2.95 (t, 2 H, $J_{4,5}$ = 9.4 Hz, H-4), 2.88 (dd, 2 H, $J_{6a,6b}$ = 13.8 Hz, $J_{5,6a}$ = 3.3 Hz, H-6a), 2.73 (dd, 2 H, $J_{5,6b}$ = 5.6 Hz, H-6b), 1.49-1.37 (m, 12 H, CH_2), 1.15 (bs, 132 H, CH_2), 0.77, 0.76 (2 t, 18 H, $J_{H,H}$ = 6.7 Hz, CH_3).

^{13}C NMR (75.5 MHz, 10:1 $CDCl_3$-CD_3OD): δ = 92.7 (C-1), 80.9 (C-3), 79.9 (C-2), 78.8 (C-4), 73.4, 73.1, 71.6 (OCH_2), 69.8 (C-5), 41.2 (C-6), 31.8-22.5 (CH_2), 13.9 (CH_3).

ESIMS: *m/z* = 1519.4 $[M + 2H]^+$, 1541.4 $[M + H + Na]^+$. Anal. Calcd for $C_{96}H_{194}Cl_2N_2O_9$: C, 72.45; H, 12.29; N, 1.76. Found: C, 72.13; H, 11.95; N, 1.51.



6,6'-Di-(2-*tert*-butoxycarbonylaminoethylthio)-2,3,4,2',3',4'-hexa-*O*-hexyl-α,α'-trehalose (82). To a solution of **76** (110 mg, 0.10 mmol) in dry DMF (12 mL), Cs_2CO_3 (94 mg, 0.29 mmol) and *tert*-butyl *N*-(2-mercaptoethyl)carbamate (49μL, 0.29 mmol) were added, under Ar atmosphere, and the reaction mixture was stirred at 60 ºC for 24 h. The reaction mixture was concentrated and the crude product was dissolved in DCM (20 mL) and washed with H_2O (2 x 30 mL). The organic phase was dried ($MgSO_4$), filtered, concentrated and purified by column chromatography (1:8 → 1:6 EtOAc-cyclohexane). Yield: 100 mg (85%). R_f = 0.26 (1:5 EtOAc-cyclohexane); $[\alpha]_D$ = +86 (*c* 1.0, DCM); IR: ν_{max} = 3350, 2928, 2855, 1710 $cm^{-1.}$

^{1}H NMR (300 MHz, $CDCl_3$): δ = 5.12 (d, 2 H, $J_{1,2}$ = 3.3 Hz, H-1), 4.99 (bs, 2 H, NHBoc), 4.01 (ddd, 2 H, $J_{4,5}$ = 9.2 Hz, $J_{5,6b}$ = 6.3 Hz, $J_{5,6a}$ = 2.7 Hz, H-5), 3.81 (m, 6 H, H-3, OCH_2), 3.70-3.46 (m, 8 H, OCH_2), 3.58 (t, 2 H, $J_{2,3}$ = $J_{3,4}$ = 9.2 Hz, H-3), 3.29 (q, 2 H, $J_{H,H}$ = $J_{H,NH}$ = 6.0 Hz, CH_2N), 3.23 (dd, 2 H, H-2), 3.16 (t, 2 H, H-4), 2.82 (dd, 2 H, $J_{6a,6b}$ = 13.5 Hz, H-6a), 2.72 (dd, 2 H, H-6b), 2.69 (t, 4 H, CH_2S), 1.60-1.47 (m, 12 H, CH_2), 1.43 (s, 18 H, CMe_3), 1.34-1.28 (m, 36 H, CH_2), 0.89 (m, 18 H, CH_3).

^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 155.7 (CO), 92.3 (C-1), 81.0 (C-3), 80.5 (C-2), 80.4 (C-4), 79.2 (*C*Me_3), 73.4, 73.2, 71.7 (OCH_2), 71.2 (C-5), 39.8 (CH_2S), 33.8 (C-6), 33.7 (CH_2N), 31.8, 31.7, 30.6, 30.5, 30.4, 30.2 (CH_2), 28.4 (C*Me*$_3$), 25.9, 25.8, 22.6 (CH_2), 14.0 (CH_3).

ESIMS: *m/z* = 1187.9 $[M + Na]^+$, 1203.8 $[M + K]^+$. Anal. Calcd for $C_{62}H_{120}N_2O_{13}S_2$: C, 63.88; H, 10.38; N, 2.40. Found: C, 63.69; H, 10.21; N, 5.19.

6,6'-Di-(2-*tert*-butoxycarbonylaminoethylthio)-2,3,4,2',3',4'-hexa-*O*-tetradecyl-α,α'-trehalose (83). To a solution of **77** (277 mg, 0.13 mmol) in dry DMF (15 mL), Cs_2CO_3 (119 mg, 0.364 mmol) and *tert*-butyl *N*-(2-mercaptoethyl)carbamate (61μL, 0.364 mmol) were added, under Ar atmosphere, and the reaction mixture was stirred at 60 ºC for 24 h. The solvent was evaporated and the crude product was dissolved in DCM (20 mL) and washed with H_2O (2 x 30 mL). The organic phase was dried ($MgSO_4$), filtered, concentrated and purified by column chromatography (1:8 EtOAc-cyclohexane). Yield: 236 mg (99%). R_f = 0.53 (1:5 EtOAc-cyclohexane); $[\alpha]_D$ = +49 (*c* 1.0, DCM).

^{1}H NMR (300 MHz, $CDCl_3$): δ = 5.12 (d, 2 H, $J_{1,2}$ = 3.4 Hz, H-1), 4.99 (bs, 2 H, NHBoc), 4.01 (ddd, 2 H, $J_{4,5}$ = 9.6 Hz, $J_{5,6b}$ = 6.1 Hz, $J_{5,6a}$ = 2.6 Hz, H-5), 3.81 (m, 4 H, OCH_2), 3.69-3.46 (m, 8 H, OCH_2), 3.58 (t, 2 H, $J_{2,3}$ = $J_{3,4}$= 9.6 Hz, H-3), 3.30 (bq, 4 H, $J_{H,H}$ = $J_{H,NH}$ = 6.0 Hz, CH_2N), 3.23 (dd, 2 H, H-2), 3.17 (t, 2 H, H-4), 2.83 (dd, 2 H, $J_{6a,6b}$ = 13.7 Hz, H-6a), 2.72 (dd, 2 H, H-6b), 2.69 (t, 4 H, CH_2S), 1.60-1.51 (m, 12 H, CH_2), 1.44 (s, 18 H, CMe_3), 1.26 (bs, 132 H, CH_2), 0.88 (t, 18 H, $J_{H,H}$ = 6.9 Hz, CH_3).

^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 155.7 (CO), 92.3 (C-1), 81.0 (C-3), 80.5 (C-2), 80.4 (C-4), 79.3 (CMe_3), 73.5, 73.2, 71.6 (OCH_2), 71.2 (C-5), 39.8 (CH_2S), 33.8 (C-6), 33.7 (CH_2N), 31.9, 30.7, 30.5, 30.3, (CH_2), 29.7 (CMe_3), 29.4, 28.4, 26.3, 26.2, 22.7 (CH_2), 14.1 (CH_3).

ESIMS: *m/z* = 1861.5 $[M + Na]^+$. Anal. Calcd for $C_{110}H_{216}N_2O_{13}S_2$: C, 71.84; H, 11.84; N, 1.52; S, 3.49. Found: C, 71.90; H, 11.72; N, 1.44; S, 3.38.

6,6'-Di-(2-aminoethylthio)-2,3,4,2',3',4'-hexa-*O*-hexyl-α,α'-trehalose dihydrochloride (84). Treatment of **82** (0.10 g, 0.09 mmol) with 1:1 TFA-DCM (2 mL) and freeze-drying from 10:1 H_2O-0.1 N HCl solution afforded **84**. Yield: 90 mg

(quantitative). R_f = 0.72 (10:1:1CH_3CN-H_2O-NH_4OH); $[\alpha]_D$ = +90.3 (*c* 1.0, DCM); IR: ν_{max} = 3300, 2928, 2859, 1099 cm^{-1}.

^1H NMR (300 MHz, $CDCl_3$): δ = 8.13 (bs, 6 H, NH_3^+), 5.20 (d, 2 H, $J_{1,2}$ = 3.2 Hz, H-1), 3.98 (dt, 2 H, $J_{4,5}$ = 9.5 Hz, $J_{5,6a}$ = $J_{5,6b}$ = 4.3 Hz, H-5), 3.79 (m, 4 H, OCH_2), 3.69-3.43 (m, 8 H, OCH_2), 3.56 (t, 2 H, $J_{2,3}$ = $J_{3,4}$ = 9.5 Hz, H-3), 3.25 (dd, 2 H, H-2), 3.18 (bs, 4 H, CH_2N), 3.11 (t, 2H, H-4), 2.97 (bd, 2 H, $J_{6a,6b}$ = 14.0 Hz, H-6a), 2.82 (m, 6 H, CH_2S, H-6b), 1.59-1.50 (m, 12 H, CH_2), 1.28 (m, 36 H, CH_2), 0.88 (m, 18 H, CH_3).

^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 91.9 (C-1), 80.9 (C-3), 80.2 (C-2), 80.0 (C-4), 73.5, 73.4, 71.8 (OCH_2), 71.0 (C-5), 39.4 (CH_2N), 34.4 (C-6), 31.8 (CH_2S), 31.7, 30.6, 30.4, 30.2, 25.9, 25.8, 22.6, 22.5 (CH_2), 14.0 (CH_3).

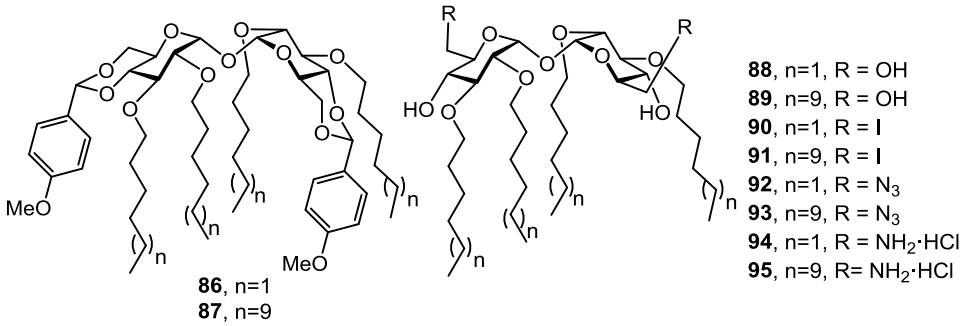
ESIMS: *m/z* = 965.9 $[M - 2Cl]^+$. Anal. Calcd for $C_{52}H_{102}N_2O_9S_2 \cdot 2HCl$: C, 60.14; H, 10.29; N, 2.70; S, 6.17. Found: C, 59.86; H, 10.02; N, 2.41; S, 5.88.

6,6'-Di-(2-aminoethylthio)-2,3,4,2',3',4'-hexa-*O*-tetradecyl-α,α'-trehalose dihydrochloride (85). Treatment of **83** (90 mg, 0.05 mmol) with 1:1 TFA-DCM (1 mL) and freeze-drying from 10:1 H_2O-0.1 N HCl solution afforded **85**. Yield: 83 mg (quantitative). R_f = 0.21 (EtOAc); $[\alpha]_D$ = +61.2 (*c* 1.0, DCM).

^1H NMR (300 MHz, $CDCl_3$): δ = 6.39 (bs, 6 H, NH_3^+), 5.21 (d, 2 H, $J_{1,2}$ = 3.5 Hz, H-1), 3.97 (dt, 2 H, $J_{4,5}$ = 9.6 Hz, $J_{5,6a}$ = $J_{5,6b}$ = 4.7 Hz, H-5), 3.79 (m, 4 H, OCH_2), 3.69-3.43 (m, 8 H, OCH_2), 3.56 (t, 2 H, $J_{2,3}$ = $J_{3,4}$ = 9.6 Hz, H-3), 3.25 (dd, 2 H, H-2), 3.18 (t, 4 H, $J_{H,H}$ = 6.3 Hz, CH_2N), 3.11 (t, 2H, H-4), 3.07-2.94 (m, 4 H, H-6a, H-6b), 2.82 (m, 4 H, CH_2S), 1.58-1.49 (m, 12 H, CH_2), 1.26 (bs, 132 H, CH_2), 0.88 (m, 18 H, $J_{H,H}$ = 7.0 Hz, CH_3).

^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 91.9 (C-1), 80.9 (C-3), 80.2 (C-2), 80.0 (C-4), 73.5, 73.4, 71.8 (OCH_2), 71.0 (C-5), 39.5 (CH_2N), 34.6 (C-6), 31.9 (CH_2S), 31.8-22.7 (CH_2), 14.1 (CH_3).

ESIMS: *m/z* = 1639.4 $[M - 2\ Cl]^+$. Anal. Calcd for $C_{100}H_{200}N_2O_9S_2 \cdot 2\ HCl$: C, 70.17; H, 11.90; N, 1.64; S, 3.75. Found: C, 69.82; H, 11.77; N, 1.39; S, 3.41.



4,6:4',6'-Di-*O*-(4-methoxybenzylidene)-2,3,2',3'-tetra-*O*-hexyl-α,α'-trehalose (**86**). To a solution of α,α'-trehalose (1.0 g, 2.92 mmol) in DMF (5 mL), camphorsulfonic acid (CSA, 20 mg cat.) and anisaldehyde dimethylacetal (1.1 mL, 5.84 mmol) were added and the resulting mixture was stirred for 40 min, concentrated and quenched by addition of saturated aqueous $NaHCO_3$ solution $NaHCO_3$ (20 mL) and stirring for an additional hour. The precipitate was filtered, washed with cold saturated aqueous solution of $NaHCO_3$ (20 mL) and triturated with hexane. Then, the solid (0.896 g, 1.55 mmol) was dissolved in DMF (15 mL), NaH (60% in mineral oil, 165 mg, 18.60 mmol) was carefully added in small portions. 1-Bromohexane (2.6 mL, 18.60 mmol) was added dropwise and the resulting mixture was stirred at 60 ºC overnight. After cooling to rt, the reaction was quenched with MeOH (1 mL) and the solution was stirred for 20 min. Solvents were then evaporated and the residue diluted with EtOAc (25 mL) and citric acid (satd aq soln, 20 mL) was added. The layers were separated and the organic layer was washed with H_2O (3 x 20 mL), dried ($MgSO_4$), evaporated and purified by flash column chromatography (1:9 EtOAc-cyclohexane). Yield: 808 mg (57%). R_f = 0.31 (1:8 EtOAc-cyclohexane); $[\alpha]_D$ = +43 (*c* 1.0, DCM); IR: ν_{max} = 2928, 2860, 1244, 1089, 988, 828 cm^{-1}.

^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.42, 6.88 (2 d, 8 H, $^3J_{H,H}$ = 8.8 Hz, A_2X_2, aromatics), 5.49 (s, 2 H, PhC*H*), 5.14 (d, 2 H, $J_{1,2}$ = 3.9 Hz, H-1), 4.18 (m, 4 H, H-5, OCH_2), 3.85-3.56 (m, 6 H, OCH_2), 3.81 (s, 6 H, $PhOCH_3$), 3.77 (t, 2 H, $J_{2,3} = J_{3,4}$ = 9.4 Hz, H-3), 3.66-3.59 (m, 4 H, H-6a, H6b), 3.49 (t, 2 H, $J_{4,5}$ = 9.4 Hz, H-4), 3.36 (dd, 2 H, H-2),

1.64-1.54 (m, 8 H, CH_2), 1.38-1.25 (m, 24 H, CH_2), 0.87, 0.86 (2 t, 12 H, $^3J_{H,H}$ = 6.8 Hz, CH_3).

^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 159.9, 130.2, 127.4, 113.4 (Ph), 101.2 (Ph*C*H), 94.5 (C-1), 82.1 (C-4), 80.0 (C-2), 78.1 (C-3), 73.4 (OCH_2), 72.0 (C-6), 69.1 (OCH_2), 62.7 (C-5), 55.2 (OCH_3), 31.7, 30.4, 30.1, 25.8, 22.6, 22.5 (CH_2), 14.0 (CH_3).

ESIMS: *m/z* = 937.5 $[M + Na]^+$. Anal. Calcd for $C_{52}H_{82}O_{13}$: C, 68.24; H, 9.03. Found: C, 68.30; H, 8.89.

4,6:4',6'-Di-*O*-(4-methoxybenzylidene)-2,3,2',3'-tetra-*O*-tetradecyl-α,α'-trehalose (**87**). To a solution of α,α'-trehalose (1.0 g, 2.92 mmol) in DMF (5 mL), camphorsulfonic acid (CSA, 20 mg cat.) and anisaldehyde dimethylacetal (1.1 mL, 5.84 mmol) were added and the resulting mixture was stirred for 40 min, concentrated and quenched by addition of saturated aqueous $NaHCO_3$ solution $NaHCO_3$ (20 mL) and stirring for an additional hour. The precipitate was filtered, washed with cold saturated aqueous solution of $NaHCO_3$ (20 mL) and triturated with hexane. Then, the solid (1.5 g, 2.592 mmol) was dissolved in DMF (25 mL), NaH (95% in mineral oil, 0.747 g, 31.11 mmol) was carefully added in small portions. 1-Bromotetradecane (9.25 mL, 31.11 mmol) was added dropwise and the resulting mixture was stirred at 60 ºC overnight. After cooling to rt, the reaction was quenched with MeOH (2.5 mL) and the solution was stirred for 20 min. Solvents were then evaporated and the residue diluted with EtOAc (50 mL) and citric acid (satd aq soln, 40 mL) was added. The layers were separated and the organic layer was washed with H_2O (3 x 40 mL), dried ($MgSO_4$), evaporated and purified by flash column chromatography (cyclohexane → 1:20 → 1:9 EtOAc-cyclohexane). Yield: 2.58 g (73%). R_f = 0.43 (1:9 EtOAc-cyclohexane); $[\alpha]_D$ = +35 (*c* 1.0, DCM); IR: ν_{max} = 2914, 2845, 1249, 1114, 979, 819 cm^{-1}.

^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.42, 6.88 (2 d, 8 H, $^3J_{H,H}$ = 9.1 Hz, A_2X_2, aromatics), 5.49 (s, 2 H, PhC*H*), 5.13 (d, 2 H, $J_{1,2}$ = 3.8 Hz, H-1), 4.18 (m, 4 H, H-5, OCH_2), 3.84-

3.56 (m, 6 H, OCH_2), 3.80 (s, 6 H, $PhOCH_3$), 3.76 (t, 2 H, $J_{2,3} = J_{3,4} = 9.5$ Hz, H-3), 3.66-3.59 (m, 4 H, H-6a, H6b), 3.48 (t, 2 H, $J_{4,5} = 9.4$ Hz, H-4), 3.35 (dd, 2 H, H-2), 1.62-1.54 (m, 8 H, CH_2), 1.25 (bs, 88 H, CH_2), 0.88 (2 t, 12 H, $^3J_{H,H} = 6.8$ Hz, CH_3).

^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 159.9, 130.2, 127.4, 113.4 (Ph), 101.2 (Ph*C*H), 94.6 (C-1), 82.2 (C-4), 80.0 (C-2), 78.2 (C-3), 73.4 (OCH_2), 72.0 (C-6), 69.1 (OCH_2), 62.7 (C-5), 55.2 (OCH_3), 31.9, 30.5, 30.1, 29.7, 29.6, 29.4, 26.2, 26.1, 22.7 (CH_2), 14.1 (CH_3).

ESIMS: *m/z* = 1386 $[M + Na]^+$. Anal. Calcd for $C_{84}H_{146}O_{13}$: C, 73.96; H, 10.79. Found: C, 74.11; H, 10.86.

2,3,2',3'-tetra-*O*-hexyl-α,α'-trehalose (**88**). To a solution of **86** (0.41 g, 0.45 mmol) in a mixture of 2:1 Et_2O-DCM (15 mL), under Ar atmosphere, $AlCl_3$ (0.720 g, 5.40 mmol) in Et_2O (10 mL) were added dropwise, and the resulting mixture was refluxed for 4 h. After cooling to rt, EtOAc (150 mL) and H_2O (150 mL) were added. The organic layer was separated, washed with brine (3 x 100 mL), dried ($MgSO_4$), evaporated and purified by column chromatography of the residue (1:2 → 2:1 → 4:1 EtOAc-cyclohexane → EtOAc). Yield: 251 mg (82%). $R_f = 0.41$ (2:1 EtOAc-cyclohexane); $[\alpha]_D = +112$ (*c* 1.1, DCM); IR: $\nu_{max} = 3301, 2933, 2850, 1113, 1046, 998$ cm^{-1}.

^{1}H NMR (300 MHz, $CDCl_3$): δ = 5.14 (d, 2 H, $J_{1,2} = 3.3$ Hz, H-1), 3.97-3.88 (m, 4 H, H-5, OCH_2), 3.83-3.72 (m, 4 H, H-6a, H-6b), 3.67-3.40 (m, 6 H, OCH_2), 3.57 (t, 1 H, $J_{2,3} = J_{3,4} = 9.1$ Hz, H-3), 3.45 (t, 1 H, $J_{4,5} = 9.1$ Hz, H-4), 3.27 (dd, 1 H, H-2), 2.79 (bs, 1 H, OH-4), 2.26 (bs, 1 H, OH-6), 1.61-1.50 (m, 8 H, CH_2), 1.36-1.27 (m, 24 H, CH_2), 0.88, 0.87 (2 t, 12 H, $^3J_{H,H} = 6.4$ Hz, CH_3).

^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 94.2 (C-1), 81.7 (C-2), 80.2 (C-3), 73.4, 71.2 (OCH_2), 71.2 (C-5), 70.0 (C-4), 62.1 (C-6), 31.7, 30.4, 30.0, 25.8, 25.7, 22.6 (CH_2), 14.0 (CH_3).

ESIMS: *m/z* = 701.4 $[M + Na]^+$. Anal. Calcd for $C_{36}H_{70}O_{11}$: C, 63.69; H, 10.39. Found: C, 63.59; H, 10.31.

2,3,2',3'-tetra-*O*-tetradecyl-α,α'-trehalose (**89**). To a solution of **87** (1.74 g, 0.450 mmol) in a mixture of 2:1 Et_2O-DCM (40 mL), under Ar atmosphere, $AlCl_3$ (2.1 g, 15.31 mmol) in Et_2O (20 mL) were added dropwise, and the resulting mixture was refluxed for 4 h. After cooling to rt, EtOAc (300 mL) and H_2O (300 mL) were added. The organic layer was separated, washed with brine (3 x 200 mL), dried ($MgSO_4$), evaporated and purified by column chromatography of the residue (1:4 → 1:2 → 1:1 EtOAc-cyclohexane). Yield: 0.589 g (41%). R_f = 0.18 (1:2 EtOAc-cyclohexane); $[\alpha]_D$ = +71 (*c* 1.0, DCM); IR: ν_{max} = 3301, 2916, 2849, 1094, 986 cm^{-1}.

^{1}H NMR (300 MHz, $CDCl_3$): δ = 5.15 (d, 2 H, $J_{1,2}$ = 3.4 Hz, H-1), 4.00-3.86 (m, 4 H, H-5, OCH_2), 3.84-3.74 (m, 4 H, H-6a, H-6b), 3.66-3.41 (m, 6 H, OCH_2), 3.57 (t, 2 H, $J_{2,3}$ = $J_{3,4}$ = 9.2 Hz, H-3), 3.46 (t, 2 H, $J_{4,5}$ = 9.2 Hz, H-4), 3.27 (dd, 1 H, H-2), 2.58 (bs, 2 H, OH-4), 2.03 (bs, 2 H, OH-6), 1.62-1.52 (m, 8 H, CH_2), 1.25 (bs, 88 H, CH_2), 0.88 (t, 12 H, $^3J_{H,H}$ = 6.5 Hz, CH_3).

^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 93.7 (C-1), 80.7 (C-2), 80.3 (C-3), 73.4, 71.2 (OCH_2), 71.2 (C-5), 70.3 (C-4), 62.3 (C-6), 31.9, 30.5, 30.1, 29.7, 29.6, 29.3, 26.2, 26.2, 22.6 (CH_2), 14.1 (CH_3).

ESIMS: *m/z* = 1149.9 $[M + Na]^+$. Anal. Calcd for $C_{68}H_{134}O_{11}$: C, 72.42; H, 11.98. Found: C, 72.11; H, 12.03.

6,6'-Dideoxy-2,3,2',3'-tetra-*O*-hexyl-6,6'-diiodo-α,α'-trehalose (90). To a solution of **88** (215 mg, 0.32 mmol) in toluene (5 mL), TPP (294 mg, 1.12 mmol) and imidazole (142 mg, 2.08 mmol) were added. Iodine (283 mg, 1.024 mmol) was added in portions and the resulting solution was stirred at 70 ºC for overnight. After cooling at rt, saturated aqueous $NaHCO_3$ (20 mL) was added and stirred 5 min. Additional iodine was added and

the mixture was stirred for 10 min. Then 10% aqueous $Na_2S_2O_3$ was added to remove the iodine excess. The organic layer was separated, washed with H_2O (3 x 20 mL), dried ($MgSO_4$), filtered, concentrated and purified by column chromatography (1:9 → 1:6 EtOAc-cyclohexane). Yield: 238 mg (83%). R_f = 0.45 (1:6 EtOAc-cyclohexane); $[\alpha]_D$ = +66 (*c* 1.0, DCM); IR: ν_{max} = 3461, 2928, 2855, 1094, 993 cm^{-1}.

^{1}H NMR (300 MHz, $CDCl_3$): δ = 5.26 (d, 2 H, $J_{1,2}$ = 3.4 Hz, H-1), 3.91 (m, 2 H, OCH_2), 3.72-3.46 (m, 12 H, H-5, H-3, H-6a, OCH_2), 3.37-3.28 (m, 6 H, H-6b, H-4, H-2), 2.46 (d, 1 H, $J_{4,OH}$ = 2.9 Hz, OH-4), 1.61-1.51 (m, 8 H, CH_2), 1.37-1.25 (m, 24 H, CH_2), 0.89, 0.88 (2 t, 12 H, $^3J_{H,H}$ = 6.5 Hz, CH_3).

^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 92.7 (C-1), 80.3 (C-3), 80.2 (C-2), 73.9, 73.4 (OCH_2), 71.7 (C-5), 69.6 (C-4), 31.7, 30.6, 30.4, 30.2, 25.8, 25.7, 22.6 (CH_2), 14.0 (CH_3), 7.8 (C-6).

ESIMS: *m/z* = 921.2 $[M + Na]^+$. Anal. Calcd for $C_{36}H_{68}I_2O_9$: C, 48.11; H, 7.63. Found: C, 48.19; H, 7.70.

6,6'-Dideoxy-2,3,2',3'-tetra-*O*-tetradecyl-6,6'-diiodo-α,α'-trehalose (91). To a solution of **89** (565 mg, 0.501 mmol) in toluene (24 mL), TPP (459 mg, 1.75 mmol) and imidazole (222 mg, 3.256 mmol) were added. Iodine (0.407 g, 1.603 mmol) was added in portions and the resulting solution was stirred at 70 ºC for overnight. After cooling at rt, saturated aqueous $NaHCO_3$ (100 mL) was added and the mixture was stirred 5 min. Additional iodine was added and the mixture was stirred for 10 min. Then 10% aqueous $Na_2S_2O_3$ was added to remove the iodine excess. The organic layer was separated, washed with H_2O (3 x 50 mL), dried ($MgSO_4$), filtered, concentrated and purified by column chromatography (1:9 EtOAc-cyclohexane). Yield: 674 mg (quantitative). R_f = 0.84 (1:6 EtOAc-cyclohexane); $[\alpha]_D$ = +51 (*c* 1.0, DCM); IR: ν_{max} = 3428, 2914, 2845, 1070 cm^{-1}.

^{1}H NMR (300 MHz, $CDCl_3$): δ = 5.27 (d, 2 H, $J_{1,2}$ = 3.3 Hz, H-1), 3.91 (m, 2 H, OCH_2), 3.72-3.46 (m, 10 H, H-5, H-6a, OCH_2), 3.60 (t, 2 H, $J_{2,3}$ = $J_{3,4}$ = 9.4 Hz, H-3), 3.37-3.28 (m, 6 H, H-6b, H-4, H-2), 2.40 (d, 1 H, $J_{4,OH}$ = 2.5 Hz, OH-4), 1.61-1.54 (m, 8 H, CH_2), 1.26 (bs, 88 H, CH_2), 0.88 (t, 12 H, $^3J_{H,H}$ = 6.8 Hz, CH_3).

^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 92.7 (C-1), 80.3 (C-3), 80.2 (C-2), 74.0, 73.4 (OCH_2), 71.7 (C-5), 69.6 (C-4), 31.9, 30.5, 30.3, 29.7, 29.4, 26.2, 26.1, 22.7 (CH_2), 14.1 (CH_3), 7.8 (C-6).

ESIMS: *m/z* = 1369.6 $[M + Na]^+$. Anal. Calcd for $C_{68}H_{132}I_2O_9$: C, 60.61; H, 9.87. Found: C, 60.82; H, 9.94.

6,6'-Diazido-6,6'-dideoxy-2,3,2',3'-tetra-*O*-hexyl-α,α'-trehalose (92). Compound **92** was obtained by treatment of **90** (0.339 g, 0.377 mmol) with NaN_3 (147 mg, 1.26 mmol) in DMF (5 mL) and purification by column chromatography (1:15 → 1:8 EtOAc-cyclohexane). Yield: 243 mg (88%). R_f = 0.39 (1:8 EtOAc-cyclohexane); $[\alpha]_D$ = -96 (*c* 1.0, DCM); IR: ν_{max} = 2104, 1085 cm^{-1}.

^{1}H NMR (300 MHz, $CDCl_3$): δ = 5.18 (d, 2 H, $J_{1,2}$ = 3.4 Hz, H-1), 4.07 (dt, 2 H, $J_{4,5}$ = 9.3 Hz, $J_{5,6a}$ = $J_{5,6b}$ = 3.6 Hz, H-5), 3.90 (m, 2 H, OCH_2), 3.62 (m, 4 H, OCH_2), 3.51-3.88 (m, 8 H, OCH_2, H-4, H-6a, H-6b), 3.53 (t, 2 H, $J_{2,3}$ = $J_{3,4}$ = 9.3 Hz, H-3), 3.29 (dd, 1 H, H-2), 2.63 (bs, 2 H, OH-4), 1.62-1.50 (m, 8 H, CH_2), 1.36-1.24 (bs, 24 H, CH_2), 0.88 (m, 12 H, CH_3).

^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 93.4 (C-1), 81.6 (C-3), 80.1 (C-2), 73.4, 71.4 (OCH_2), 70.9 (C-5), 70.7 (C-4), 51.6 (C-6), 31.7, 30.4, 30.1, 25.8, 25.7, 22.6 (CH_2), 14.0 (CH_3).

ESIMS: *m/z* = 751.4 $[M + Na]^+$. Anal. Calcd for $C_{36}H_{68}N_6O_9$: C, 59.32; H, 9.40; N, 11.53. Found: C, 59.44; H, 9.52; N, 11.62.

6,6'-Diazido-6,6'-dideoxy-2,3,2',3'-tetra-*O*-tetradecyl-α,α'-trehalose (93). Compound **93** was obtained by treatment of **91** (0.229 g, 0.170 mmol) with NaN_3 (66 mg, 1.02 mmol) in DMF (5 mL) and purification by column chromatography (1:15 EtOAc-cyclohexane). Yield: 172 mg (86%). R_f = 0.39 (1:8 EtOAc-cyclohexane); $[\alpha]_D$ = 72 (*c* 1.0, DCM); IR: ν_{max} = 2099, 1113 cm^{-1}.

^1H NMR (300 MHz, $CDCl_3$): δ = 5.18 (d, 2 H, $J_{1,2}$ = 3.4 Hz, H-1), 4.07 (ddd, 2 H, $J_{4,5}$ = 9.2 Hz, $J_{5,6a}$ = 3.6 Hz, $J_{5,6b}$ = 8.2 Hz, H-5), 3.90 (m, 2 H, OCH_2), 3.62 (m, 2 H, OCH_2), 3.67-3.45 (m, 8 H, OCH_2, H-4), 3.52 (t, 2 H, $J_{2,3}$ = $J_{3,4}$ = 9.3 Hz, H-3), 3.48-3.38 (m, 4 H, H-6a, H-6b), 3.29 (dd, 1 H, H-2), 2.39 (d, 2 H, $J_{4,OH}$ = 2.3 Hz, OH-4), 1.59-1.50 (m, 8 H, CH_2), 1.24 (bs, 88 H, CH_2), 0.86 (t, 12 H, $^3J_{H,H}$ = 6.9 Hz, CH_3).

^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 93.5 (C-1), 80.6 (C-3), 80.2 (C-2), 73.4, 71.4 (OCH_2), 70.9 (C-5), 70.7 (C-4), 51.7 (C-6), 31.9, 30.5, 30.2, 29.7, 26.2, 26.1, 22.7 (CH_2), 14.1 (CH_3).

ESIMS: *m/z* = 1200.8 $[M + Na]^+$. Anal. Calcd for $C_{68}H_{132}N_6O_9$: C, 69.34; H, 11.30; N, 7.14. Found: C, 69.50; H, 11.31; N, 7.02.

6,6'-Diamino-6,6'-dideoxy-2,3,2',3'-tetra-*O*-hexyl-α,α'-trehalose dihydrochloride (94). To a solution of **92** (251 mg, 0.34 mmol) in THF (22.5 mL), TPP (357 mg, 1.36 mmol) was added and the mixture was stirred at rt for 15 min. Then NH_4OH (2.5 mL) was added and the solution was stirred overnight at 50 ºC. The mixture was concentrated, the resulting residue purified by column chromatography (50:10:1→ 40:10:1 DCM-MeOH-H_2O → MeOH) and freeze-dried. Yield: 220 mg (86%). R_f = 0.16 (50:10:1 DCM-MeOH-H_2O); $[\alpha]_D$ = +91 (*c* 0.9 in 9:1 DCM-MeOH); IR: ν_{max} = 3450, 2927, 2853, 1107, 1004 cm^{-1}.

^1H NMR (300 MHz, CD_3OD): δ = 5.24 (d, 2 H, $J_{1,2}$ = 3.2 Hz, H-1), 3.95 (dt, 2 H, $J_{4,5}$ = 10.0 Hz, $J_{5,6a}$ = $J_{5,6b}$ = 3.9 Hz, H-5), 3.80 (t, 4 H, $J_{H,H}$ = 6.6 Hz, OCH_2), 3.72-3.57 (m, 4 H, OCH_2), 3.58 (t, 2 H, $J_{2,3}$ = $J_{3,4}$ = 9.3 Hz, H-3), 3.38 (dd, 2 H, H-4), 3.31 (dd, 2 H, H-2),

3.05 (m, 4 H, H-6a, H-6b), 1.65-1.55 (m, 8 H, CH_2), 1.44-1.31 (m, 24 H, CH_2), 0.93 (t, 12 H, $J_{H,H}$ = 6.6 Hz, CH_3).

^{13}C NMR (75.5 MHz, CD_3OD): δ = 94.8 (C-1), 82.3 (C-3), 81.1 (C-2), 74.5, 72.7 (OCH_2), 71.8 (C-4), 71.5 (C-5), 42.0 (C-6), 33.0, 31.5, 31.4, 27.0, 26.9, 23.7 (CH_2), 14.4 (CH_3).

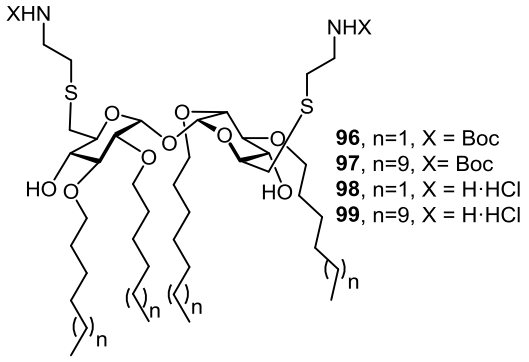
ESIMS: *m/z* = 677.4 $[M - 2\ HCl]^+$. Anal. Calcd for $C_{36}H_{74}Cl_2N_2O_9$: C, 57.66; H, 9.95; N, 3.74. Found: C, 57.59; H, 9.88; N, 2.60.

6,6'-Diamino-6,6'-dideoxy-2,3,2',3'-tetra-*O*-tetradecyl-α,α'-trehalose dihydrochloride (95). To a solution of **93** (166 mg, 0.141 mmol) in THF (20 mL), TPP (148 mg, 0.564 mmol) was added and the mixture was stirred at rt for 15 min. Then NH_4OH (2 mL) was added and the solution was stirred overnight at 50 ºC. The mixture was concentrated, the resulting residue purified by column chromatography (70:10:1→ 40:10:1 DCM-MeOH-H_2O) and freeze-dried. Yield: 158 mg (quantitative). R_f = 0.29 (50:10:1 DCM-MeOH-H_2O); $[\alpha]_D$ = +67 (*c* 1.0 in 5:1 DCM-MeOH); IR: ν_{max} = 3450, 2922, 2853, 1111, 1013 cm^{-1}.

^{1}H NMR (300 MHz, 5:1 $CDCl_3$-CD_3OD): δ = 4.98 (d, 2 H, $J_{1,2}$ = 3.5 Hz, H-1), 3.74 (m, 2 H, H-5), 3.65 (t, 4 H, $J_{H,H}$ = 6.9 Hz, OCH_2), 3.51-3.38 (m, 6 H, H-3, OCH_2), 3.24 (dd, 2 H, $J_{3,4}$ = 9.2 Hz, $J_{4,5}$ = 10.0 Hz, H-4), 3.13 (dd, 2 H, $J_{2,3}$ = 9.2 Hz, H-2), 2.88 (dd, 2 H, $J_{6a,6b}$ = 13.3 Hz, $J_{5,6a}$ = 3.7 Hz, H-6a), 2.79 (dd, 2 H, $J_{5,6b}$ = 3.7 Hz, H-6b), 1.51-1.37 (m, 8 H, CH_2), 1.14 (bs, 88 H, CH_2), 0.76 (t, 12 H, $J_{H,H}$ = 6.9 Hz, CH_3).

^{13}C NMR (75.5 MHz, 5:1 $CDCl_3$-CD_3OD): δ = 93.3 (C-1), 80.6 (C-3), 79.7 (C-2), 73.4, 71.5 (OCH_2), 70.5 (C-4), 70.5 (C-5), 41.3 (C-6), 31.7, 30.2, 30.0, 29.5, 29.1, 25.9, 22.4 (CH_2), 13.7 (CH_3).

ESIMS: *m/z* = 1125.9 $[M - 2\ HCl]^+$. Anal. Calcd for $C_{68}H_{138}Cl_2N_2O_9$: C, 68.13; H, 11.60; N, 2.34. Found: C, 68.42; H, 11.86; N, 2.28.



6,6'-Di-(2-*tert*-butoxycarbonylaminoethylthio)-2,3,2',3'-tetra-*O*-hexyl-α,α'-trehalose (96). To a solution of **90** (217 mg, 0.241 mmol) in dry DMF (28 mL), Cs_2CO_3 (220 mg, 0.675 mmol) and *tert*-butyl *N*-(2-mercaptoethyl)carbamate (114 μL, 0.675 mmol) were added, under Ar atmosphere, and the reaction mixture was stirred at 60 ºC overnight. The reaction mixture was concentrated and the crude product was dissolved in DCM (40 mL) and washed with H_2O (2 x 60 mL). The organic phase was dried ($MgSO_4$), filtered and concentrated. The residue was purified by column chromatography (1:6 EtOAc-cyclohexane). Yield: 240 mg (quantitative) R_f = 0.47 (1:2 EtOAc-cyclohexane); $[\alpha]_D$ = +50 (*c* 1.0, DCM); IR: ν_{max} = 3354, 2923, 2856, 1696, 1163 cm^{-1}.

^{1}H NMR (300 MHz, $CDCl_3$): δ = 5.18 (d, 2 H, $J_{1,2}$ = 3.4 Hz, H-1), 4.99 (bs, 2 H, NHBoc), 4.04 (ddd, 2 H, $J_{4,5}$ = 9.4 Hz, $J_{5,6a}$ = 2.9 Hz, $J_{5,6b}$ = 6.3 Hz, H-5), 3.89 (m, 2 H, OCH_2), 3.70-3.39 (m, 6 H, OCH_2), 3.55 (t, 2 H, $J_{2,3}$ = $J_{3,4}$ = 9.2 Hz, H-3), 3.43 (t, 2 H, H-4), 3.30 (m, 4 H, CH_2N), 3.28 (dd, 2 H, H-2), 2.90 (dd, 2 H, $J_{6a,6b}$ = 14.0 Hz, H-6a), 2.76 (m, 4 H, H-6b, OH-4), 2.67 (t, 4 H, $J_{H,H}$ = 6.5 Hz, CH_2S), 1.60-1.53 (m, 8 H, CH_2), 1.43 (s, 18 H, CMe_3), 1.34-1.24 (m, 24 H, CH_2), 0.88 (m, 12 H, CH_3).

^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 155.8 (CO), 92.5 (C-1), 80.6 (C-3), 80.2 (C-2), 79.4 (CMe_3), 73.3, 72.1, (OCH_2), 71.4 (C-5), 71.3 (C-4), 38.5 (CH_2S), 33.7 (C-6, CH_2N), 31.7, 31.3, 30.4, 30.1 (CH_2), 28.4 (CMe_3), 25.8, 22.6 (CH_2), 14.0 (CH_3).

ESIMS: *m/z* = 1019.6 $[M + Na]^+$. Anal. Calcd for $C_{50}H_{96}N_2O_{13}S_2$: C, 60.21; H, 9.70; N, 2.81; S, 6.43. Found: C, 60.40; H, 9.80; N, 2.80; S, 6.49.

6,6'-Di-(2-aminoethylthio)-2,3,2',3'-tetra-*O*-hexyl-α,α'-trehalose dihydrochloride (98). Treatment of **96** (139 mg, 0.139 mmol) with 1:1 TFA-DCM (2 mL) was carried out as indicated in general methods. Yield: 100 mg (83%). R_f = 0.29 (10:2:1 CH_3CN-H_2O-NH_4OH); $[\alpha]_D$ = +77 (*c* 1.0, MeOH); IR: ν_{max} = 3399, 2923, 2860, 1129 cm^{-1}.

^1H NMR (300 MHz, CD_3OD): δ = 5.21 (d, 2 H, $J_{1,2}$ = 3.4 Hz, H-1), 4.03 (ddd, 2 H, $J_{4,5}$ = 9.3 Hz, $J_{5,6b}$ = 6.8 Hz, $J_{5,6a}$ = 2.3 Hz, H-5), 3.77 (m, 6 H, OCH_2), 3.56 (t, 2 H, $J_{2,3}$ = $J_{3,4}$ = 9.3 Hz, H-3), 3.55 (m, 2 H, OCH_2), 3.47 (t, 2H, H-4), 3.27 (dd, 2 H, H-2), 3.14 (t, 4 H, $J_{H,H}$ = 6.8 Hz, CH_2S), 2.95 (bd, 2 H, $J_{6a,6b}$ = 14.0 Hz, H-6a), 2.89 (m, 4 H, CH_2N), 2.78 (dd, 2 H, H-6b), 1.63-1.56 (m, 8 H, CH_2), 1.41-1.29 (t, 24 H, $J_{H,H}$ = 7.0 Hz, CH_2), 0.91 (m, 12 H, CH_3).

^{13}C NMR (75.5 MHz, CD_3OD): δ = 93.5 (C-1), 82.1 (C-3), 81.5 (C-2), 74.6, 73.9 (OCH_2), 73.9 (C-5), 72.9 (C-4), 40.0 (CH_2N), 34.5 (C-6), 33.0 (CH_2S), 33.0, 31.6, 31.5, 31.4, 27.0, 26.9, 23.7 (CH_2), 14.4 (CH_3).

ESIMS: *m/z* = 797.5 $[M - 2HCl]^+$. Anal. Calcd for $C_{40}H_{80}N_2O_9S_2 \cdot 2$ HCl: C, 55.22; H, 9.50; N, 3.22; S, 7.37. Found: C, 55.10; H, 9.48; N, 3.15; S, 7.19.

6,6'-Di-(2-*tert*-butoxycarbonylaminoethylthio)-2,3,2',3'-tetra-*O*-tetradecyl-α,α'-trehalose (97). To a solution of **91** (284 mg, 0.211 mmol) in dry DMF (25 mL), Cs_2CO_3 (193 mg, 0.591 mmol) and *tert*-butyl *N*-(2-mercaptoethyl)carbamate (100 μL, 0.591 mmol) were added, under Ar atmosphere, and the reaction mixture was stirred at 60 ºC overnight. The reaction mixture was concentrated and the crude product was dissolved in DCM (30 mL) and washed with H_2O (2 x 50 mL). The organic phase was dried ($MgSO_4$), filtered and concentrated. The residue was purified by column chromatography (1:6 EtOAc-cyclohexane). Yield: 305 mg (quantitative). R_f = 0.60 (1:2 EtOAc-cyclohexane); $[\alpha]_D$ = +51 (*c* 1.0, DCM); IR: ν_{max} = 3350, 2918, 2848, 1692, 1170 cm^{-1}.

^1H NMR (300 MHz, $CDCl_3$): δ = 5.18 (d, 2 H, $J_{1,2}$ = 3.3 Hz, H-1), 4.96 (bs, 2 H, NHBoc), 4.05 (ddd, 2 H, $J_{4,5}$ = 9.2 Hz, $J_{5,6b}$ = 6.3 Hz, $J_{5,6a}$ = 2.9 Hz, H-5), 3.89 (m, 2 H,

OCH_2), 3.72-3.43 (m, 8 H, OCH_2, H-4), 3.55 (t, 2 H, $J_{2,3} = J_{3,4}$ = 9.2 Hz, H-3), 3.30 (m, 4 H, CH_2N), 3.29 (dd, 2 H, H-2), 2.91 (dd, 2 H, $J_{6a,6b}$ = 13.7 Hz, H-6a), 2.76 (dd, 2 H, H-6b), 2.69 (t, 4 H, $J_{H,H}$ = 6.3 Hz, CH_2S), 2.61 (bs, 2 H, OH-4), 1.70-1.54 (m, 8 H, CH_2), 1.44 (s, 18 H, CMe_3), 1.25 (bs, 88 H, CH_2), 0.88 (t, 12 H, $J_{H,H}$ = 6.8 Hz, CH_3).

^{13}C NMR (125.7 MHz, $CDCl_3$): δ = 155.9 (CO), 92.5 (C-1), 80.5 (C-3), 80.2 (C-2), 79.4 (CMe_3), 73.3, 72.1, (OCH_2), 71.4 (C-5), 71.3 (C-4), 39.6 (CH_2S), 33.7 (C-6), 33.5 (CH_2N), 31.9, 30.5, 30.2, 30.1 (CH_2), 29.7 (CMe_3), 29.3, 28.4, 26.9, 26.2, 26.1, 22.7 (CH_2), 14.1 (CH_3).

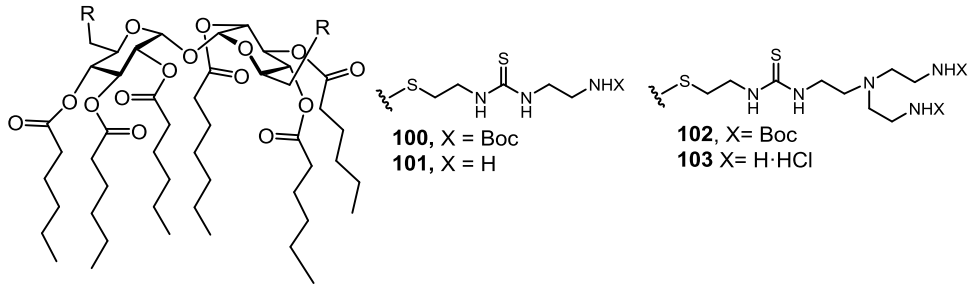
ESIMS: *m/z* = 1468.0 $[M + Na]^+$. Anal. Calcd for $C_{82}H_{160}N_2O_{13}S_2$: C, 68.10; H, 11.15; N, 1.94; S, 4.43. Found: C, 67.95; H, 11.28; N, 1.84; S, 4.61.

6,6'-Di-(2-aminoethylthio)-2,3,2',3'-tetra-*O*-tetradecyl-α,α'-trehalose dihydrochloride (99). Treatment of **97** (140 mg, 0.097 mmol) with 1:1 TFA-DCM (2 mL) was carried out as indicated in general methods. Yield: 120 mg (quantitative). R_f = 0.26 (45:5:3 EtOAc-EtOH-H_2O); $[\alpha]_D$ = +59 (*c* 1.0, DCM); IR: ν_{max} = 3429, 2923, 2848, 1135 cm^{-1}.

^{1}H NMR (500 MHz, $CDCl_3$): δ = 5.29 (d, 2 H, $J_{1,2}$ = 3.0 Hz, H-1), 4.02 (m, 2 H, H-5), 3.93 (m, 2 H, OCH_2), 3.65 (m, 4 H, OCH_2), 3.57 (t, 2 H, $J_{2,3} = J_{3,4}$ = 9.5 Hz, H-3), 3.50 (m, 2 H, OCH_2), 3.42 (t, 2 H, $J_{4,5}$ = 9.5 Hz, H-4), 3.35 (dd, 2 H, H-2), 3.17 (m, 4 H, CH_2N), 2.97 (m, 4 H, CH_2S), 2.95 (bd, 2 H, $J_{6a,6b}$ = 13.8 Hz, H-6a), 2.81 (m, 2 H, H-6b), 1.61-1.55 (m, 8 H, CH_2), 1.29 (bs, 88 H, CH_2), 0.90 (t, 12 H, $J_{H,H}$ = 6.9 Hz, CH_3).

^{13}C NMR (125.7 MHz, $CDCl_3$): δ = 91.9 (C-1), 80.5 (C-3), 79.9 (C-2), 73.5, 71.9 (OCH_2), 71.6 (C-5), 71.4 (C-4), 39.0 (CH_2N), 36.5 (C-6), 34.0 (CH_2S), 31.9, 31.4, 31.3, 30.4, 30.2, 29.7, 29.6, 29.4, 26.1, 22.7 (CH_2), 14.1 (CH_3).

ESIMS: *m/z* = 1246.0 $[M - 2\ HCl]^+$. Anal. Calcd for $C_{72}H_{146}Cl_2N_2O_9S_2$: C, 65.56; H, 11.16; N, 2.12; S, 4.86. Found: C, 65.30; H, 10.89; N, 1.96; S, 4.64.



[6-(2-(*N´*-(2-(*N*-*tert*-Butoxycarbonylamino)ethyl)thioureido)ethylthio)-2,3-di-*O*-hexanoyl]-α,α'-trehalose (100). To a solution of **70** (140 mg, 0.125 mmol) and Et_3N (51.8 μL, 0.374 mmol) in DCM (12 mL), *tert*-butyl *N*-(2-isothiocyanoethyl) carbamate (76 mg, 0.374 mmol) was added and the reaction mixture was stirred at rt for 36 h. The reaction mixture was washed with aqueous diluted HCl (2 x 20 mL), dried ($MgSO_4$), filtered and concentrated. The residue was purified by column chromatography (1:1 EtOAc-cyclohexane). Yield: 185 mg (73%). R_f = 0.63 (2:1 EtOAc-cyclohexane); $[\alpha]_D$ = + 62.1 (*c* 1, DCM); UV (DCM): λ_{max} = 248 nm (ε_{mM} 73.1); IR: ν_{max} = 2957, 1749, 1685 cm^{-1}.

^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.01, 6.91 (bs, 4 H, NHCS), 5.47 (t, $J_{3,4}$ = 9.5 Hz, 2 H, H-3), 5.4 (bd, 2 H, H-1), 5.16 (bs, 2 H, NHBoc), 5.05 (dd, 2 H, $J_{1,2}$ = 3.9 Hz, H-2), 4.95 (t, 2 H, H-4), 3.94 (m, 2 H, H-5), 3.62 (m, 4 H, SCH_2CH_2NHCS), 3.56 (bs, 4 H, CH_2NHCS), 3.29 (m, 4 H, CH_2NHBoc), 2.84 (t, 4 H, $J_{H,H}$ = 7.0 Hz, SCH_2CH_2NHCS), 2.58 (m, 4 H, H-6a, H-6b), 2.34-2.31 (m, 12 H, $H\text{-}2a_{Hex}$, $H\text{-}2b_{Hex}$), 1.55 (m, 12 H, $H\text{-}3_{Hex}$), 1.42 (s, 18 H, CMe_3), 1.28 (m, 24 H, $H\text{-}4_{Hex}$, $H\text{-}5_{Hex}$), 0.88 (t, 9 H, $J_{H,H}$ = 6.5 Hz, $H\text{-}6_{Hex}$).

^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 173-172.3 (CO ester), 157.8 (CO carbamate), 91.3 (C-1), 80.1 (CMe_3),72.1 (C-5), 71.0 (C-4), 69.8 (C-2), 69.6 (C-3), 45.5 (CH_2NHCS), 43.7 (SCH_2CH_2NHCS), 39.9 (CH_2NHBoc), 32.7 (CH_2S, C-6), 34.2-34.0 ($C\text{-}2_{Hex}$), 31.1 ($C\text{-}4_{Hex}$), 28.5 (CMe_3) 24.3 ($C\text{-}3_{Hex}$), 22.1 ($C\text{-}5_{Hex}$), 13.8 ($C\text{-}6_{Hex}$).

ESIMS: *m/z* = 1487.5 $[M + Cl]^-$. Anal. Calcd for $C_{68}H_{120}N_6O_{19}S_4$: C, 56.17; H, 8.32; N, 5.78; S, 8.82. Found: C, 56.24; H, 8.26; N, 5.59; S, 8.51.

[6-(2-(*N´*-(2-Aminoethyl)thioureido)ethylthio)-2,3-di-*O*-hexanoyl]-α,α'-trehalose dihydrochloride (101). Treatment of **100** (119 mg, 0.082 mmol) with 1:1 TFA-DCM (1.6 mL) as indicated in general methods yielded quantitatively **101**. Yield: 119 mg. $[\alpha]_D$ = + 62.1 (*c* 1, MeOH); UV (MeOH): λ_{max} = 245 nm (ε_{mM} 24.1); IR: ν_{max} = 2955, 1750 cm^{-1}.

^{1}H NMR (500 MHz, CD_3OD): δ 5.51 (t, 2 H, $J_{2,3} = J_{3,4}$ = 9.5 Hz, H-3), 5.41 (d, 2 H, H-1), 5.12 (dd, 2 H, $J_{1,2}$ = 4.2 Hz, H-2), 5.07 (t, 2 H, $J_{4,5}$ = 9.5 Hz, H-4), 4.00 (m, 2 H, H-5), 3.83 (m, 4 H, NC*H*$_2$CH$_2$NHCS), 3.66 (bs, 4 H, C*H*$_2$CH$_2$S), 3.16 (m, 4 H, NCH$_2$C*H*$_2$NHCS), 2.77 (m, 4 H, CH$_2$C*H*$_2$S), 2.75 (m, 4 H, H-6a, H-6b), 2.42-2.26 (m, 12 H, H-2$_{Hex}$), 1.65-1.54 (m, 12 H, H-3$_{Hex}$), 1.32 (m, 24 H, H-4$_{Hex}$, H-5$_{Hex}$), 0.92 (m, 18 H, H-6$_{Hex}$).

^{13}C NMR (125 MHz, CD_3OD): δ = 173.2-172.4 (CO), 90.8 (C-1), 71.1 (C-5), 70.0 (C-4), 69.8 (C-3), 69.6 (C-2), 44.3 (*C*H$_2$CH$_2$S), 40.8 (*C*H$_2$CH$_2$NHCS), 39.4 (CH$_2$*C*H$_2$NHCS), 32.7 (CH$_2$S), 31.8 (C-6), 33.6, 33.8, 31.2 (C-2$_{Hex}$), 31.1 (C-4 $_{Hex}$), 24.2 (C-3$_{Hex}$), 22.1 (C-5$_{Hex}$), 13.1 (C-6$_{Hex}$).

ESIMS: *m/z* = 1253.7 $[M]^+$. Anal. Calcd for $C_{58}H_{104}N_6O_{15}S_4 \cdot 2$ HCl: C, 52.51; H, 8.05; N, 6.33; S, 9.67. Found: C, 52.34; H, 7.89; N, 6.14; S, 9.33.

Bis[6-(2-(*N*'-(2-(*N,N*-di-(2-(*N*-*tert*-butoxycarbonylamino)ethyl)amino)-ethyl)thioureido)ethylthio)-2,3-di-*O*-hexanoyl]-α,α'-trehalose (102). To a solution of **70** (180 mg, 0.16 mmol) and Et_3N (89 μL, 0.64 mmol, 2 equiv) in DCM (9 mL) 2-[Bis[2-(*tert*-butoxycarbonylamino)ethyl]amine]ethylisothiocyanate **167** (150 mg, 0.38 mmol, 2 equiv) was added and the reaction mixture was stirred at rt for 48 h. The reaction mixture was washed with aqueous diluted HCl (2 x 20 mL), dried ($MgSO_4$), filtered, concentrated and the residue was purified by column chromatography (1:1 → 2:1 → 3:1 EtOAc-cyclohexane). Yield: 126 mg (50%). R_f = 0.31 (2:1 EtOAc-cyclohexane); $[\alpha]_D$ = +

58.2 (*c* 1, DCM); UV (MeOH): λ_{max} = 247 nm (ε_{mM} 27.9); IR: ν_{max} = 2957, 1751, 1685 cm^{-1}.

^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.51, 7.04 (2 bs, 4 H, NHCS), 5.47 (t, 2 H, $J_{2,3} = J_{3,4}$ = 9.8 Hz, H-3), 5.31 (d, 2 H, $J_{1,2}$ = 4.0 Hz, H-1), 5.07 (dd, 2 H, H-2), 5.00 (t, 2 H, $J_{4,5}$ = 9.8 Hz, H-4), 3.96 (m, 2 H, H-5), 3.71 (m, 4 H, S*C*$H_2$$CH_2$NHCS), 3.57 (bs, 4 H, N$CH_2$*C*$H_2$NHCS) 3.17 (m, 4 H, *C*$H_2$NHBoc), 2.77 (t, 4 H, $J_{H,H}$ = 6.7 Hz, CH_2*C*H_2S), 2.64-2.61 (m, 12 H, H-6a, H-6b, *C*$H_2$$CH_2$NHBoc), 2.26 (m, 12 H, H-2_{Hex}), 1.56 (m, 12 H, H-3_{Hex}), 1.42 (s, 18 H, CMe_3), 1.28 (m, 24 H, H-4_{Hex}, H-5_{Hex}), 0.88 (t, 18 H, $J_{H,H}$ = 6.7 Hz, H-6_{Hex}).

^{13}C NMR (75 MHz, $CDCl_3$): δ = 183.5 (CS), 172.1 (CO), 91.2 (C-1), 71.5 (C-4), 71.2 (C-5), 69.9 (C-2), 69.7 (C-3), 55.3 (*C*$H_2$$CH_2$NHBoc), 54.3 (*C*$H_2$$CH_2$S), 42.2 (N$CH_2$*C*$H_2$NHCS), 38.7 (*C*$H_2$NHBoc), 34.1 (C-$2_{Hex}$), 32.9 (C-6), 31.3 (C-$4_{Hex}$), 24.3 (C-$3_{Hex}$), 22.3 (C-$5_{Hex}$), 14.1 (C-$6_{Hex}$).

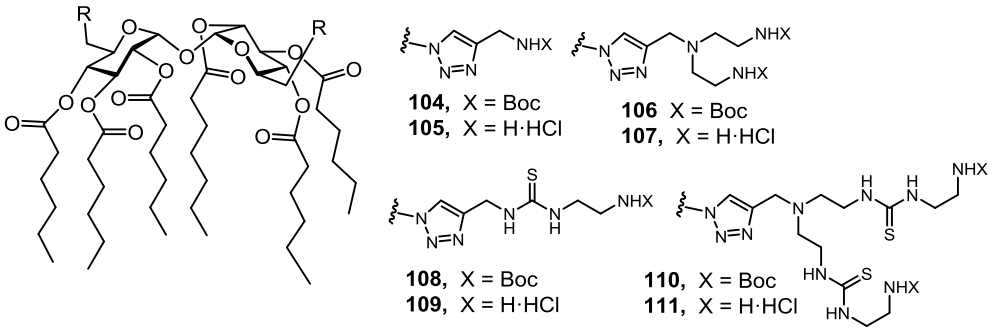
ESIMS: *m/z* = 1848.9 $[M + Na]^+$. Anal. Calcd for $C_{86}H_{156}N_{10}O_{23}S_4$: C, 56.55; H, 8.61; N, 7.67; S, 7.02. Found: C, 56.36; H, 8.44; N, 7.50; S, 6.78.

Bis[6-(2-(N'-(2-(*N,N*-bis-(2-aminoethyl)amino)ethyl)thioureido)ethylthio)-2,3-di-*O*-hexanoyl]-α,α'-trehalose tetrahydrochloride (103). Treatment of **102** (110 mg, 0.07 mmol) with 1:1 TFA-DCM (1.6 mL) as indicated in general methods yielded quantitatively **103**. Yield: 111 mg. $[\alpha]_D$ = + 59.14 (*c* 1.0, MeOH); UV (MeOH): λ_{max} = 243 nm (ε_{mM} 39.1); IR: ν_{max} = 2956, 1751 cm^{-1}.

^{1}H NMR (300 MHz, CD_3OD): δ = 5.51 (t, 2 H, $J_{2,3} = J_{3,4}$ = 9.5 Hz, H-3), 5.41 (d, 2 H, $J_{1,2}$ = 4.2 Hz, H-1), 5.13 (dd, 2 H, H-2), 5.00 (t, 2 H, $J_{4,5}$ = 9.5 Hz, H-4), 4.00 (m, 2 H, H-5), 3.66 (m, 8 H, NHCSC$H_2$$CH_2$N, *C*$H_2$$CH_2$S), 3.17 (t, 4 H, $J_{H,H}$ = 5.9 Hz, *C*$H_2$$NH_3$Cl), 2.83 (t, 4 H, *C*$H_2$$CH_2$$NH_3$Cl), 2.77 (t, 4 H, CH_2*C*H_2S), 2.76 (m, 4 H, H-6a, H-6b), 2.31 (m, 12 H, H-2_{Hex}), 1.59 (m, 12 H, H-3_{Hex}), 1.32 (m, 24 H, H-4_{Hex}, H-5_{Hex}), 0.91 (t, 18 H, $J_{H,H}$ = 6.5 Hz, H-6_{Hex}).

^{13}C NMR (75.5 MHz, CD_3OD): δ = 184.3 (CS), 174.2-174 (CO), 92.5 (C-1), 72.6 (C-5), 72.4 (C-4), 71.6 (C-3), 71.2 (C-2), 52.8 (C*H*$_2$CH$_2$NH$_3$Cl), 45.2 (NHCS*C*H$_2$CH$_2$N) 42.4 (*C*H$_2$CH$_2$S), 38.7 (*C*H$_2$NH$_3$Cl), 35.2 (C-2$_{Hex}$), 34.1 (C-6), 32.6 (C-4$_{Hex}$), 25.9 (C-3$_{Hex}$), 23.7 (C-5$_{Hex}$), 14.4 (C-6$_{Hex}$).

ESIMS: *m/z* = 713 $[M]^{2+}$, 1489 $[M + Cu]^+$. Anal. Calcd for $C_{66}H_{116}N_{10}O_{15}S_4 \cdot 4$ HCl: C, 50.69; H, 7.73; N, 8.96; S, 8.20. Found: C, 50.13; H, 7.90; N, 8.67; S, 7.81.



[6,6'-(Di-*tert*-butoxycarbonylaminomethyl-1*H*-1,2,3-triazol-1-yl)-6,6'-dideoxy-2,3,4,2',3',4'-hexa-*O*-hexanoyl-α,α'-trehalose (104). To a solution of **68** (250 mg, 0.255 mmol) and *N-tert*-butoxycarbonylpropargylamine **170** (88 mg, 0.561 mmol) in ^{t}BuOH-H_2O (9:1, 15 mL), **Si-BPA·Cu$^+$** (17 mg) was added and the reaction mixture was refluxed for 36 h at 85 ºC. The catalyst was filtered off and the solvent was removed. The residue was purified by column chromatography (1:1 → 2:1 EtOAc-cyclohexane). Yield: 334 mg (quantitative). R_f = 0.73 (2:1 EtOAc-cyclohexane); $[\alpha]_D$ = +56.7 (*c* 1.0, DCM); IR: ν_{max} = 2957, 1752, 1714, 735 cm^{-1}.

^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.52 (s, 2 H, =CH), 5.45 (t, 2 H, $J_{2,3} = J_{3,4}$ = 9.8 Hz , H-3), 5.30 (bs, 2 H, NHBoc), 4.97 (dd, 2 H, $J_{1,2}$ = 4.0 Hz, H-2), 4.87 (t, 2 H, $J_{4,5}$ = 9.8 Hz, H-4), 4.83 (d, 2 H, H-1), 4.48 (bd, 2 H, $J_{6a,6b}$ = 13.7 Hz, H-6a), 4.37 (d, 4 H, $J_{H,H}$ = 6.0 Hz, CH_2-triazole), 4.12 (dd, 2 H, $J_{5,6b}$ = 8.8 Hz, H-6b), 4.07 (m, 2 H, H-5), 2.25 (m, 12 H, H-

2_{Hex}), 1.55 (m, 12 H, H-3_{Hex}), 1.46 (s, 18 H, CMe_3), 1.29 (m, 24 H, H-4_{Hex}, H-5_{Hex}), 0.89 (m, 18 H, H-6_{Hex}).

^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 173.5-173.2 (CO ester), 155.5 (CO carbamate), 146.0 (C-4 triazole), 122.3 (C-5 triazole), 91.7 (C-1), 79.5 (*C*Me_3), 69.9 (C-3, C-5), 69.8 (C-4), 69.3 (C-2), 50.7 (C-6), 36.4 (CH_2-triazole), 34.3 (C-2_{Hex}), 31.9 (C-4_{Hex}), 28.7 (C*Me*$_3$) 24.6 (C-3_{Hex}), 22.6 (C-5_{Hex}), 14.1 (C-6_{Hex}).

ESIMS: *m/z* = 1313.4 $[M + Na]^+$. Anal. Calcd for $C_{64}H_{106}N_8O_{19}$: C, 59.52; H, 8.27; N, 8.68. Found: C, 59.61; H, 8.33; N, 8.84.

6,6'-*(*4-Aminomethyl-1*H*-1,2,3-triazol-1-yl)-6-deoxy-2,3,4,2',3',4'-hexa-*O*-hexanoyl)-α,α'-trehalose dihydrochloride (105). Treatment of **104** (300 mg, 0.23 mmol) with 1:1TFA-DCM (4 mL) as indicated in general methods yielded **105**. Yield: 260 mg (97%). $[\alpha]_D$ = +48.9 (*c* 1.0, MeOH); IR: ν_{max} = 2956, 1751, 1464, 1026 cm^{-1}.

^{1}H NMR (300 MHz, CD_3OD): δ = 8.05 (s, 2 H, =CH), 5.49 (t, 2 H, $J_{3,4} = J_{2,3}$ = 9.8 Hz, H-3), 5.06 (dd, 2 H, $J_{1,2}$ = 4.0 Hz, H-2), 4.96 (d, 2 H, H-1), 4.95 (t, 2 H, $J_{4,5}$ = 9.8 Hz, H-4), 4.63 (dd, 2 H, $J_{6a,6b}$ = 14.6 Hz, $J_{5,6a}$ = 2.6 Hz, H-6a), 4.53 (dd, 2 H, $J_{5,6b}$ = 7.9 Hz, H-6b), 4.27 (bs, 4 H, C*H*$_2$$NH_2$), 4.24 (m, 1 H, H-5), 2.32 (m, 12 H, H-2_{Hex}), 1.59 (m, 12 H, H-3_{Hex}), 1.33 (m, 24 H, H-4_{Hex}, H-5_{Hex}), 0.93 (m, 18 H, H-6_{Hex}).

^{13}C NMR (75.5 MHz, CD_3OD): δ = 174.5-173.6 (CO), 141.5 (C-4 triazole) 127.1 (C-5 triazole), 92.7 (C-1), 71.2 (C-2), 70.6 (C-3), 70.4 (C-4), 70.3 (C-5), 51.5 (C-6), 35.2 (CH_2-triazole), 34.8 (C-2_{Hex}), 32.1 (C-4_{Hex}), 25.8 (C-3_{Hex}), 22.6 (C-5_{Hex}), 14.2 (C-6_{Hex}).

ESIMS: *m/z* = 1091.4 $[M]^+$. Anal. Calcd for $C_{54}H_{92}N_8O_{15}$: C, 55.71; H, 7.96; N, 9.62. Found: C, 55.48; H, 7.77; N, 9.97.

6,6'-[-4-(2,2-Bis-*tert*-butoxycarbonylamino)ethylaminomethyl)-1*H*-1,2,3-triazol-1-yl)]-6,6'-dideoxy-2,3,4,2'3',4'-hexa-*O*-hexanoyl-α,α'-trehalose (106). To a solution of **68** (310 mg, 0.315 mmol) and bis[2-*tert*-butoxycarbonylamino)ethyl]propargylamine **169** (240 mg, 0.73 mmol) in ^{t}BuOH-H_2O (9:1, 15 mL), **Si-BPA·Cu$^+$** (21 mg) was added and

the reaction mixture was refluxed for 36 h at 85 ºC. The catalyst was filtered off and the solvent was removed. The residue was purified by column chromatography (2:1 → 3:1 EtOAc-cyclohexane). Yield: 543 mg (91%). R_f = 0.36 (3:1 EtOAc-cyclohexane); $[\alpha]_D$ = +57.0 (*c* 1.0, DCM); IR: ν_{max} = 2958, 2359, 2341, 1751, 1700 cm^{-1}.

^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.57 (s, 2 H, =CH), 5.47 (t, 2 H, $J_{2,3} = J_{3,4}$ = 9.8 Hz, H-3), 5.20 (bs, 4 H, NHBoc), 4.93 (dd, 2 H, $J_{1,2}$ = 3.8 Hz, H-2), 4.86 (t, 2 H, $J_{4,5}$ = 10.0 Hz, H-4), 4.77 (d, 2 H, H-1), 4.46 (bd, 2 H, $J_{6a,6b}$ = 14.0 Hz, H-6a), 4.25 (dd, 2 H, $J_{5,6b}$ = 8.7 Hz, H-6b), 4.12 (m, 2 H, H-5), 3.83 (bs, 4 H, CH_2-triazole), 3.19 (bd, 8 H, C*H*$_2$NHBoc), 2.55 (t, 8 H, $J_{H,H}$ = 6.0 Hz, C*H*$_2$CH$_2$NHBoc), 2.27 (m, 12 H, H-2_{Hex}), 1.58 (s, 36 H, CMe_3), 1.44 (m, 12 H, H-3_{Hex}), 1.30 (m, 24 H, H-4_{Hex}, H-5_{Hex}), 0.89 (m, 18 H, H-6_{Hex}).

^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 174.2-173.0 (CO ester), 156.2 (CO carbamate), 143.9 (C-4 triazole), 124.3 (C-5 triazole), 91.4 (C-1), 79.1 (*C*Me$_3$), 69.5 (C-4), 69.3 (C-3), 69.2 (C-5), 68.8 (C-2), 53.1 (*C*H$_2$CH$_2$NHBoc), 50.6 (C-6), 48.0 (CH_2-triazole), 38.4 (*C*H$_2$NHBoc), 34.1 (C-2_{Hex}), 31.7 (C-4_{Hex}), 28.5 (C*Me$_3$*), 24.6 (C-3_{Hex}), 22.6 (C-5_{Hex}), 13.8 (C-6_{Hex}).

ESIMS: *m/z* = 1686.6 $[M + Na]^+$. Anal. Calcd for $C_{82}H_{142}N_{12}O_{23}$: C, 59.18; H, 8.60; N, 10.10. Found: C, 59.20; H, 8.51; N, 10.15.

6,6'[4-(2,2-Diaminoethylaminomethyl)-1*H*-1,2,3-triazol-1-yl]-6,6'-dideoxy-2,3,4,2',3',4'-hexa-*O*-hexanoyl]-α,α'-trehalose tetrahydrochloride (107). Treatment of **106** (560 mg, 0.336 mmol) with 1:1 TFA-DCM (6 mL) as indicated in general methods yielded **107**. Yield: 483 mg (quantitative). $[\alpha]_D$ = +22.1 (*c* 1.0, MeOH); IR: ν_{max} = 2956, 2356, 1753, 1676, 721 cm^{-1}.

^{1}H NMR (300 MHz, CD_3OD): δ = 8.33 (s, 2 H, =CH), 5.51 (t, 2 H, $J_{3,4} = J_{2,3}$ = 9.7 Hz, H-3), 5.08 (dd, 2 H, $J_{1,2}$ = 3.9 Hz, H-2), 5.01 (t, 2 H, $J_{4,5}$ = 9.7 Hz, H-4), 4.95 (d, 2 H, H-1), 4.65 (dd, 2 H, $J_{6a,6b}$ = 14.5 Hz, $J_{5,6a}$ = 3.0 Hz, H-6a), 4.56 (dd, 2 H, $J_{5,6b}$ = 8.1 Hz, H-6b), 4.28 (bs, 4 H, CH_2-triazole), 4.18 (ddd, 2 H, H-5), 3.41 (bt, 8 H, $J_{H,H}$ = 6.0 Hz, C*H*$_2$NH$_2$),

3.22 (bt, 8 H, C*H*$_2$CH$_2$NH$_2$), 2.31 (m, 12 H, H-2$_{Hex}$), 1.59 (m, 12 H, H-3$_{Hex}$), 1.33 (m, 24 H, H-4$_{Hex}$, H-5$_{Hex}$), 0.93 (m, 18 H, H-6$_{Hex}$).

^{13}C NMR (75.5 MHz, CD_3OD): δ = 174.2-173.2 (CO), 141.1 (C-4 triazole), 128.8 (C-5 triazole), 91.9 (C-1), 71.0 (C-4), 70.6 (C-3), 70.5 (C-5), 70.4 (C-2), 51.8 (C-6), 51.6 (*C*H$_2$CH$_2$NH$_2$), 47.9 (CH$_2$-triazole), 37.2 (CH$_2$NH$_2$), 35.1-34.8 (C-2$_{Hex}$), 32.5-32.4 (C-4$_{Hex}$), 25.6-25.5 (C-3$_{Hex}$), 23.4 (C-5$_{Hex}$), 14.3 (C-6$_{Hex}$).

ESIMS: *m/z* = 1263.5 $[M]^+$; 632.0 $[M]^{2+}$. Anal. Calcd for $C_{62}H_{110}N_{12}O_{15}$·4 HCl: C, 52.83; H, 8.15; N, 11.93. Found: C, 52.87; H, 8.04; N, 11.74.

6,6'-[Di-4-(2-N'-(2-(N-*tert*-butoxycarbonyl)aminoethyl)thioureidomethyl)-1*H*-1,2,3-triazol-1-yl]-6,6'-dideoxy-2,3,4,2',3',4'-hexa-*O*-hexanoyl-α,α'-trehalose (108). To a solution of **105** (140 mg, 0.12 mmol) and Et_3N (37 μL, 0.36 mmol) in DCM (10 mL), *tert*-butyl *N*-(2-isothiocyanoethyl)carbamate **168** (73 mg, 0.36 mmol) was added and the reaction mixture was stirred overnight at rt. The reaction mixture was washed with aqueous diluted HCl (3 x 10 mL) and the organic phase was dried ($MgSO_4$), filtered, and concentrated. The residue was purified by column chromatography (2:1 → 3:1 EtOAc-cyclohexane). Yield: 121 mg (67%). R_f = 0.40 (3:1 EtOAc-cyclohexane); $[\alpha]_D$ = +59.8 (*c* 1.0, DCM); UV (DCM): λ_{max} = 249 nm (ε_{mM} 53.9); IR: ν_{max} = 2959, 1752, 1701, cm^{-1}.

^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.80 (s, 2 H, =CH), 7.07 (bs, 4 H, NHCS), 5.39 (t, 2 H, $J_{3,4}$ = $J_{2,3}$ = 9.7 Hz , H-3), 5.29 (bs, 2 H, NHBoc), 4.98-4.76 (m, 8 H, H-1, H-4, CH$_2$-triazole), 4.62 (m, 2 H, H-2), 4.47 (d, 2 H, $J_{6a,6b}$ = 14.0 Hz, H-6a), 4.28 (dd, 2 H, $J_{5,6b}$ = 8.0 Hz, H-6b), 3.97 (bt, 2 H, H-5), 3.60 (bs, 4 H, C*H*$_2$CH$_2$NHBoc), 3.31 (m, 4 H, C*H*$_2$NHBoc), 2.22 (m, 12 H, H-2$_{Hex}$), 1.53 (m, 12 H, H-3$_{Hex}$), 1.39 (s, 18 H, CMe$_3$), 1.27 (m, 24 H, H-4$_{Hex}$, H-5$_{Hex}$), 0.86 (m, 18 H, H-6$_{Hex}$).

^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 173.2-172.5 (CO ester), 156.2 (CO carbamate), 141.5 (C-4 triazole), 124.3 (C-5 triazole), 96.7 (C-1), 79.7 (*C*Me$_3$), 69.6-68.7 (C-2, C-3,

C-4, C-5), 50.3 (C-6), 40.0 (*C*$H_2$$CH_2$NHBoc, CH_2-triazole), 39.5 (CH_2NHBoc), 33.9 (C-2_{Hex}), 31.2 (C-4_{Hex}), 28.3 (C*Me*$_3$), 24.3 (C-3_{Hex}), 22.2 (C-5_{Hex}), 13.1 (C-6_{Hex}).

ESIMS: *m/z* = 1517.4 $[M + Na]^+$. Anal. Calcd for $C_{70}H_{118}N_{12}O_{19}S_2$: calcd. C, 56.20; H, 7.95; N, 11.24; S, 4.29. Found: C, 55.95; H, 7,84; N, 11.04; S, 4.19.

6,6'-[Di-4-(2-N'-(2-aminoethyl)thioureidomethyl)-1*H*-1,2,3-triazol-1-yl]-6,6'-dideoxy-2,3,4,2',3',4'-hexa-*O*-hexanoyl-α,α'-trehalose dihydrochloride (109). Treatment of **108** (111 mg, 0.074 mmol) with 1:1 TFA-DCM (2 mL) as indicated in general methods yielded **109**. Yield: 103 mg (quantitative). $[\alpha]_D$ = +38.2 (*c* 1.0, MeOH); UV (MeOH): λ_{max} = 243 nm (ε_{mM} 16.9); IR: ν_{max} = 2956, 2862, 1752, 1675 721 cm^{-1}.

^{1}H NMR (300 MHz, CD_3OD): δ = 7.92 (s, 2 H, =CH), 5.44 (t, 2 H, $J_{2,3}$ = $J_{3,4}$ = 9.7 Hz, H-3), 5.08 (dd, 2 H, $J_{1,2}$ = 4.2 Hz, H-2), 4.95 (t, 2 H, $J_{4,5}$ = 9.7 Hz, H-4), 4.82 (m, 6 H, H-1, CH_2-triazole), 4.62 (dd, 2 H, $J_{6a,6b}$ = 14.0 Hz, $J_{5,6a}$ = 2.5 Hz, H-6a), 4.48 (dd, 2 H, $J_{5,6b}$ = 8.2 Hz, H-6b), 4.19 (ddd, 2 H, H-5), 3.91 (m, 4 H, C*H*$_2$$CH_2$$NH_2$), 3.21 (dt, 4 H, $J_{H,H}$ = 5.8 Hz, C*H*$_2$$NH_2$), 2.31 (m, 12 H, H-$2_{Hex}$), 1.59 (m, 12 H, H-$3_{Hex}$), 1.32 (m, 24 H, H-$4_{Hex}$, H-$5_{Hex}$), 0.93 (m, 18 H, H-$6_{Hex}$).

^{13}C NMR (75.5 MHz, CD_3OD): δ = 182.6 (CS), 176.0-174.5 (CO), 146.1 (C-4 triazole), 125.6 (C-5 triazole), 92.4 (C-1), 71.4 (C-4), 70.6 (C-3), 70.4 (C-5), 70.3 (C-2), 51.5 (C-6), 42.6 (*C*$H_2$$CH_2$$NH_2$), 40.9 ($CH_2$$NH_2$), 40.2 ($CH_2$-triazole), 35-34.8 (C-$2_{Hex}$), 32.5-32.4 (C-$4_{Hex}$), 25.7-25.4 (C-$3_{Hex}$), 23.2 (C-$5_{Hex}$), 14.3 (C-$6_{Hex}$).

ESIMS: *m/z* = 1357 $[M + Cu]^+$, 711 $[M + Cu]^{2+}$. Anal. Calcd for $C_{60}H_{102}N_{12}O_{15}S_2 \cdot 2$ HCl: C, 52.66; H, 7.66; N, 12.28; S, 4.69. Found: C, 52.71; H, 7.50; N, 12.55, 4.69.

Dendritic trehalose derivative 110. To a solution of **107** (230 mg, 0.163 mmol) and Et_3N (99 μL, 0.98 mmol) in DCM (17 mL), *tert*-butyl N-(2-isothiocyanoethyl)carbamate **168** (198 mg, 0.98 mmol) was added and the mixture was stirred at rt overnight. The solvent was removed and the residue was purified by column chromatography (3:1

EtOAc-cyclohexane → 9:1 DCM-MeOH). Yield: 232 mg (69%). R_f = 0.45 (9:1 DCM-MeOH); $[\alpha]_D$ = +30.26 (*c* 1.0, DCM); UV (DCM): λ_{max} = 247 nm (ε_{mM} 32.3); IR: ν_{max} = 3307, 2959, 1752, 1674, 733 cm^{-1}.

^{1}H NMR (300 MHz, $CDCl_3$, 303 K): δ = 7.77 (s, 2 H, =CH), 7.25, 7.10 (bs, 4 H, NHCS), 5.46 (bs, 4 H, NHBoc), 5.44 (t, 2 H, $J_{2,3} = J_{3,4}$ = 9.7 Hz, H-3), 4.91 (t, 2 H, $J_{4,5}$ = 9.7 Hz, H-4), 4.87 (bs, 2 H, H-1), 4.85 (dd, 2 H, H-2), 4.65 (bs, 4 H, CH_2-triazole), 4.54 (d, 2 H, $J_{6a,6b}$ = 14.0 Hz, H-6a), 4.34 (dd, 2 H, $J_{5,6b}$ = 9.0 Hz, H-6b), 4.07 (bs, 8 H, NCH_2CH_2NHCS), 3.97 (bt, 2 H, H-5), 3.60 (bs, 8 H, NCH_2CH_2NHCS, $NHBocCH_2CH_2NHCS$), 3.30 (m, 8 H, CH_2NHBoc), 2.29 (m, 12 H, H-2_{Hex}), 1.55 (m, 12 H, H-3_{Hex}), 1.41 (s, 36 H, CMe_3), 1.29 (m, 24 H, H-4_{Hex}, H-5_{Hex}), 0.90 (t, 18 H, $J_{H,H}$ = 6.5 Hz, H-6_{Hex}).

^{13}C NMR (75.5 MHz, $CDCl_3$, 303 K): δ = 183.1 (CS), 173.0-172.1 (CO ester), 156.7 (CO carbamate), 144.2 (C-4 triazole), 128.3 (C-5 triazole), 90.8 (C-1), 79.6 (CMe_3), 69.4 (C-2), 69.3 (C-4), 69.1(C-3), 68.8 (C-5), 53.5 (C-6), 52.3 (NCH_2CH_2NHCS), 50.4 (C-6), 48.2 (CH_2-triazole), 45.9 ($NHBocCH_2CH_2NHCS$), 40.2 (CH_2NHBoc), 33.9 (C-2_{Hex}), 31.2 (C-4_{Hex}), 28.3 (CMe_3), 24.3 (C-3_{Hex}), 22.18 (C-5_{Hex}), 13.9 (C-6_{Hex}).

ESIMS: *m/z* = 2135.5 $[M + Cu]^+$. Anal. Calcd for $C_{94}H_{166}N_{20}O_{23}S_4$: C, 54.47; H, 8.07; N, 13.52; S, 6.19. Found: C, 55.54; H, 8.01; N, 13.6; S, 5.97.

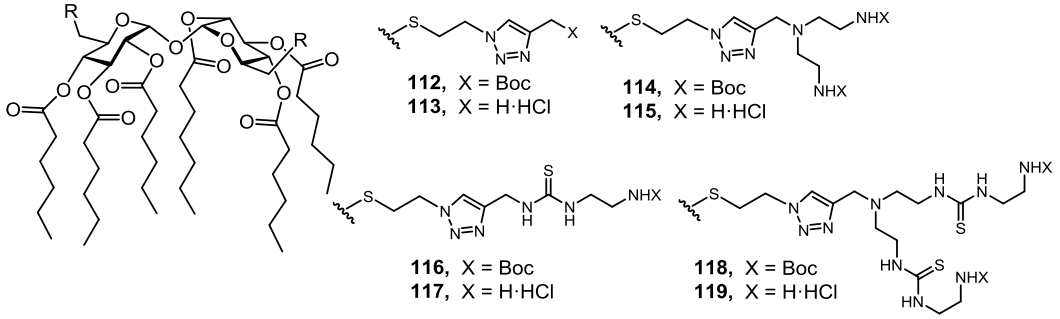
Dentritic trehalose derivative 111. Treatment of **110** (160 mg, 0.077 mmol) with 1:1 TFA-DCM (3 mL) as indicated in general methods yielded **110**. Yield: 122 mg (94%). $[\alpha]_D$ = +23.9 (*c* 0.89, MeOH); UV (MeOH): λ_{max} = 243 nm (ε_{mM} 34.3); IR: ν_{max} = 3230, 2954, 1752, 1676, 721 cm^{-1}.

^{1}H NMR (300 MHz, CD_3OD): δ = 8.43 (s, 2 H, =CH), 5.49 (t, 2 H, $J_{2,3} = J_{3,4}$ = 9.7 Hz, H-3), 5.07 (dd, 2 H, $J_{1,2}$ = 3.9 Hz, H-2), 5.03 (t, 2 H, $J_{4,5}$ = 9.7 Hz, H-4), 4.91 (d, 2 H, H-1), 4.77 (bs, 4 H, CH_2-triazole), 4.67 (dd, 2 H, $J_{5,6a}$ = 2.9 Hz, $J_{6a,6b}$ = 14.7 Hz, H-6a), 4.59 (dd, 2 H, $J_{5,6b}$ = 8.1 Hz, H-6b), 4.15 (ddd, 2 H, H-5), 4.11 (m, 8 H, H-5, $CH_2CH_2NH_2$), 3.89 (t,

8 H, $J_{H,H}$ 6.0 Hz, NCH_2CH_2NHCS), 3.56 (bs, 8 H, CH_2NH_2), 3.23 (t, 8 H, NCH_2CH_2NHCS), 2.50-2.19 (m, 12 H, H-2_{Hex}), 1.60 (m, 12 H, H-3_{Hex}), 1.33 (m, 24 H, H-4_{Hex}, H-5_{Hex}), 0.93 (m, 18 H, H-6_{Hex}).

^{13}C NMR (75.5 MHz, CD_3OD): δ = 184.5 (CS), 174.2-173.2 (CO), 136.2 (C-4 triazole), 128.1 (C-5 triazole), 90.8 (C-1), 71.1 (C-2), 70.7 (C-3), 70.6 (C-4), 70.4 (C-5), 54.5 (CH_2NH_2), 51.7 (C-6), 48.0 (CH_2-triazole), 42.6 ($NH_2CH_2CH_2NHCS$), 40.7 (NCH_2CH_2NHCS), 40.4 (CH_2NH_2), 34.9 (C-2_{Hex}), 32.5-32.4 (C-4_{Hex}), 25.6-25.5 (C-3_{Hex}), 23.4 (C-5_{Hex}), 14.3 (C-6_{Hex}).

ESIMS: *m/z* = 1733.3 $[M + Cu]^+$, 867.1 $[M + Cu]^{2+}$. Anal. Calcd for $C_{74}H_{134}N_{20}O_{15}S_4 \cdot 4$ HCl: C, 48.89; H, 7.65; N, 15.41; S, 7.05. Found: C, 48.81; H, 7.35; N, 15.10; 7.17.



6,6'-Di-(2-(4-*tert*-butoxycarbonylaminomethyl-1*H*-1,2,3-triazol-1-yl)ethyltio)-6,6'-dideoxy-2,3,4,2',3',4'-hexa-*O*-hexanoyl-α,α'-trehalose (112). To a solution of **71** (260 mg, 0.23 mmol) and *N*-(*tert*-butoxycarbonyl)propargylamine **170** (81 mg, 0.52 mmol) in 9:1 ^{t}BuOH-H_2O mixture (15 mL), **Si-BPA·Cu**$^+$ (16 mg) was added and the reaction mixture was refluxed for 24 h at 85 ºC. The catalyst was filtered off and the solvent was removed. The residue was purified by column chromatography (2:1 → 4:1 EtOAc-cyclohexane). Yield: 327 mg (quantitative). R_f = 0.50 (2:1 EtOAc-cyclohexane); $[\alpha]_D$ = + 71.8 (*c* 1.0, DCM); IR: ν_{max} = 2958, 1748, 1704, 726 cm^{-1}.

^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.50 (s, 2 H, =CH), 5.49 (t, 2 H, $J_{2,3} = J_{3,4}$ = 10 Hz, H-3), 5.26 (d, 2 H, $J_{1,2}$ = 3.7 Hz, H-1), 5.02 (dd, 2 H, H-2), 4.97 (t, 2 H, $J_{4,5}$ = 9.6 Hz, H-4), 4.47 (t, 4 H, $J_{H,H}$ = 6.5 Hz, CH_2Ntriazole), 4.39 (d, 4 H, $J_{H,H}$ = 5.8 Hz, CH_2-triazole), 3.89 (m, 2 H, H-5), 3.07 (m, 4 H, CH_2S), 2.47 (m, 4 H, H-6a, H-6b), 2.37-2.14 (m, 12 H, H-2_{Hex}), 1.55 (m, 12 H, H-3_{Hex}), 1.43 (s, 18 H, CMe_3), 1.29 (m, 24 H, H-4_{Hex}, H-5_{Hex}), 0.88 (t, 18 H, $J_{H,H}$ = 6.5 Hz, H-6_{Hex}).

^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 172.6, 172.4 (CO ester), 155.8 (CO carbamate), 131.5 (C-4 triazole), 122.2 (C-5 triazole), 91.1 (C-1), 79.5 (*C*Me_3) 71.4 (C-4), 71.0 (C-2), 69.6 (C-5), 69.5 (C-3), 50.0 (CH_2Ntriazole), 36.2 (CH_2-triazole), 34.5 (CH_2S), 34.1 (C-2_{Hex}), 33.0 (C-6), 31.3 (C-4_{Hex}), 28.2 (C*Me*$_3$), 24.5 (C-3_{Hex}), 22.2 (C-5_{Hex}), 13.9 (C-6_{Hex}).

ESIMS: *m/z* = 1433.5 $[M + Na]^+$. Anal. Calcd for $C_{68}H_{114}N_8O_{19}S_2$: C, 57.85; H, 8.14; N, 7.94; S, 4.54. Found: C, 57.76; H, 8.19; N, 7.76; S, 4.32.

6,6'-Di-(2-(4-aminomethyl-1*H*-1,2,3-triazol-1-yl)ethyltio)-6,6'-dideoxy-2,3,4,2',3',4'-hexa-*O*-hexanoyl-α,α'-trehalose dihydrochloride (113). Treatment of **112** (160 mg, 0.113 mmol) with 1:1 TFA-DCM (4 mL) at rt for 1 h. Then, the solvent was evaporated and acid traces were removed by co-evaporation with water. The residue was solved in 10:1 H_2O-HCl 0.1 N and freeze-dried. Yield: 132 mg (91%). $[\alpha]_D$ = + 51.78 (*c* 1.0, MeOH); IR: ν_{max} = 2956, 1750, 1685, 1152, 970 cm^{-1}.

^{1}H NMR (300 MHz, CD_3OD): δ = 7.15 (s, 2 H, =CH), 5.49 (t, 2 H, $J_{2,3} = J_{3,4}$ = 10.0 Hz, H-3), 5.35 (d, 2 H, $J_{1,2}$ = 3.8 Hz, H-1), 5.10 (dd, 2 H, H-2), 5.05 (t, 2 H, $J_{4,5}$ = 9.7 Hz, H-4), 4.64 (t, 4 H, $J_{H,H}$ = 7.3 Hz, CH_2N-triazole), 4.28 (s, 4 H, CH_2-triazole), 3.97 (m, 2 H, H-5), 3.15 (m, 4 H, CH_2S), 2.64 (m, 4 H, H-6a, H-6b), 2.40-2.20 (m, 12 H, H-2_{Hex}), 1.58 (m, 12 H, H-3_{Hex}), 1.31 (m, 24 H, H-4_{Hex}, H-5_{Hex}), 0.92 (t, 18 H, $J_{H,H}$ = 6.5 Hz, H-6_{Hex}).

^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 174.1, 173.8 (CO), 141.1 (C-4 triazole), 126.6 (C-5 triazole), 92.3 (C-1), 72.3 (C-4), 72.1 (C-2), 71.3 (C-5), 71.1 (C-3), 51.2 (CH_2N-triazole), 35.5 (CH_2-triazole), 34.9 (C-2_{Hex}), 34.5 (CH_2S), 34.4 (C-6), 32.3 (C-4_{Hex}), 25.5 (C-3_{Hex}), 23.5 (C-5_{Hex}), 14.1 (C-6_{Hex}).

ESIMS: *m/z* = 1233.8 $[M + Na]^+$, 1249.8 $[M + K]^+$. Anal. Calcd for $C_{58}H_{98}N_8O_{15}S_2 \cdot 2$ HCl: C, 54.23; H, 7.85; N, 8.72; S, 4.99. Found: C, 53.87; H, 7.66; N, 8.39; S, 4.60.

Dendritic trehalose derivative 114. To a solution of azide **71** (281 mg, 0.25 mmol) and 3-bis[2-*tert*-butoxycarbonylamino)ethyl]propargylamine **169** (192 mg, 0.56 mmol) in 9:1 ^{t}BuOH-H_2O mixture (15 mL), **Si-BPA·Cu**$^+$ (17 mg) was added and the reaction mixture was refluxed for 24 h at 85 ºC. The catalyst was filtered off and the solvent was removed. The residue was purified by column chromatography (3:1 EtOAc-cyclohexane → 9:1 DCM-MeOH). Yield: 448 mg (quantitative). R_f = 0.67 (9:1 DCM-MeOH); $[\alpha]_D$ = + 55.17 (*c* 1.0, DCM); IR: ν_{max} = 2959, 1748, 1699, 726 cm^{-1}.

^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.57 (s, 2 H, =CH), 5.47 (t, 2 H, $J_{2,3} = J_{3,4}$ = 9.5 Hz, H-3), 5.28 (d, 2 H, $J_{1,2}$ = 4.0 Hz, H-1), 5.17 (bs, 2 H, NHBoc), 5.02 (dd, 2 H, H-2), 4.98 (t, 2 H, $J_{4,5}$ = 9.8 Hz, H-4), 4.48 (t, 4 H, $J_{H,H}$ = 6.9 Hz, CH_2N), 3.91 (m, 2 H, H-5), 3.80 (s, 4 H, CH_2-triazole), 3.21 (bd, 8 H, C*H*$_2$NHBoc), 3.06 (m, 4 H, CH_2S), 2.55 (t, 8 H, $J_{H,H}$ = 5.6 Hz, C*H*$_2$CH$_2$NHBoc), 2.49 (m, 4 H, H-6a, H-6b), 2.38-2.13 (m, 12 H, H-2_{Hex}), 1.55 (m, 12 H, H-3_{Hex}), 1.43 (s, 36 H, CMe_3), 1.27 (m, 24 H, H-4_{Hex}, H-5_{Hex}), 0.87 (m, 18 H, H-6_{Hex}).

^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 172.5 (CO ester), 156.2 (CO carbamate), 144.4 (C-4 triazole), 123.4 (C-5 triazole), 88.2 (C-1), 79.1 (*C*Me$_3$), 71.3 (C-4), 70.9 (C-2), 69.6 (C-5), 69.4 (C-3), 53.1 (CH_2NHBoc), 50.0 (CH_2N triazole), 38.2 (CH_2-triazole), 33.6 (CH_2S), 34.1 (C-2_{Hex}), 33.1 (C-6), 31.3 (C-4_{Hex}), 28.6 (C*Me*$_3$), 24.3 (C-3_{Hex}), 22.3 (C-5_{Hex}), 13.8 (C-6_{Hex}).

ESIMS: *m/z* = 1806.8 $[M + Na]^+$. Anal. Calcd for $C_{58}H_{98}N_8O_{15}S_2$: C, 57.85; H, 8.14; N, 7.94; S, 4.54. Found: C, 57.76; H, 8.19; N, 7.76; S, 4.32.

Dendritic trehalose derivative 115. Treatment of **114** (412 mg, 0.23 mmol) with 1:1 TFA-DCM (6 mL) as described in general methods yielded **115.** Yield: 359 mg (quantitative). $[\alpha]_D$ = + 45.38 (*c* 1.0, MeOH); IR: ν_{max} = 2956, 1751, 1677 cm^{-1}.

^{1}H NMR (300 MHz, CD_3OD): δ = 8.09 (s, 2 H, =CH), 5.49 (t, 2 H, $J_{2,3} = J_{3,4}$ = 9.7 Hz, H-3), 5.36 (d, 2 H, $J_{1,2}$ = 4.0 Hz, H-1), 5.09 (dd, 2 H, H-2), 5.04 (t, 2 H, $J_{4,5}$ = 9.7 Hz, H-4), 4.61 (t, 4 H, $J_{H,H}$ = 6.5 Hz, C*H*$_2$N), 4.00 (m, 2 H, H-5), 3.90 (s, 4 H, CH_2-triazole), 3.15 (m, 12 H, CH_2S, $CH_2CH_2NH_2$), 2.81 (t, 8 H, $J_{H,H}$ = 6.0 Hz, C*H*$_2$$CH_2NH_2$), 2.63 (m, 4 H, H-6a, H-6b), 2.42-2.21 (m, 12 H, H-2$_{Hex}$), 1.58 (m, 12 H, H-3$_{Hex}$), 1.31 (m, 24 H, H-4$_{Hex}$, H-5$_{Hex}$), 0.91 (m, 18 H, H-6$_{Hex}$).

^{13}C NMR (75.5 MHz, CD_3OD): δ = 174.8, 173.8 (CO), 143.4 (C-4 triazole), 126.1 (C-5 triazole), 92.3 (C-1), 72.6 (C-4), 72.3 (C-2), 71.3 (C-5), 71.1 (C-3), 52.0 (C*H*$_2$$NH_2$), 51.2 ($CH_2$N-triazole), 47.2 ($CH_2$-triazole), 38.2 (C*H*$_2$$CH_2NH_2$), 35.1 (C-2$_{Hex}$), 34.9 ($CH_2S$), 34.0 (C-6), 32.5 (C-4$_{Hex}$), 25.6 (C-3$_{Hex}$), 23.1 (C-5$_{Hex}$), 14.0 (C-6$_{Hex}$).

ESIMS: *m/z* = 1384.0 $[M]^+$, 692.5 $[M + H]^{2+}$. Anal. Calcd for $C_{66}H_{118}N_{12}O_{15}S_2 \cdot 4$ HCl: C, 51.82; H, 8.04; N, 10.99; S, 4.19. Found: C, 51.59; H, 8.12; N, 10.68; S, 3.82.

6,6'-Di-[2-(4-(*N*'-(2-(*tert*-butoxycarbonylamino)ethyl)thioureidomethyl)-1*H*-1,2,3-triazol-1-yl)ethylthio]-6,6'-dideoxy-2,3,4,2',3',4'-hexa-*O*-hexanoyl-α,α'-trehalose (116). To a solution of **113** (146 mg, 0.11 mmol) and Et_3N (47 μL, 0.341 mmol), *tert*-butyl *N*-(2-isothiocyanoethyl)carbamate **170** (69.1 mg, 0.341 mmol) was added, and the mixture was stirred at rt, under Ar atmosphere, for 48 h. The mixture was washed with HCl 0.1 N (2 x 10 mL) and the organic phase was dried ($MgSO_4$), filtered and concentrated. The residue was purified by column chromatography (4:1 → 8:1 EtOAc-cyclohexane → 9:1 DCM-MeOH). Yield: 100 mg (54%). R_f = 0.71 (9:1 DCM-MeOH); $[\alpha]_D$ = + 10.17 (*c* 1.0, DMSO-d_6); UV (DMSO-H_2O 0.4%): λ_{max} = 249 nm (ε_{mM} 27.4); IR: ν_{max} = 3320, 2956, 1751, 1699, 968 cm^{-1}.

^{1}H NMR (300 MHz, DMSO-d_6): δ = 8.0 (s, 2 H, =CH), 7.85, 7.55 (bs, 4 H, NHCS), 6.83 (bs, 2 H, NHBoc), 5.35 (t, 2 H, $J_{2,3} = J_{3,4}$ = 9.5 Hz, H-3), 5.25 (d, 2 H, $J_{1,2}$ = 3.8 Hz, H-1), 5.04 (dd, 2 H, H-2), 5.02 (t, 2 H, $J_{4,5}$ = 9.8 Hz, H-4), 4.64 (d, 4 H, $J_{H,H}$ = 4.8 Hz, CH_2-triazole), 4.49 (t, 4 H, $J_{H,H}$ = 6.7 Hz CH_2N), 3.88 (m, 2 H, H-5), 3.42 (bs, 4 H,

C*H*$_2$NHCS), 3.03 (m, 8 H, C*H*$_2$NHBoc, CH$_2$S), 2.66 (m, 4 H, H-6a, H-6b), 2.41-2.12 (m, 12 H, H-2$_{Hex}$), 1.48 (m, 12 H, H-3$_{Hex}$), 1.37 (s, 18 H, CMe$_3$), 1.24 (m, 24 H, H-4$_{Hex}$, H-5$_{Hex}$), 0.85 (m, 18 H, H-6$_{Hex}$).

^{13}C NMR (75.5 MHz, CDCl$_3$): δ = 172.6, 172.4 (CO ester), 155.8 (CO carbamate), 122.2 (C5triazole), 91.1 (C-1), 71.4 (C-4), 71.0 (C-2), 69.6 (C-5), 69.5 (C-3), 50.0 (*C*H$_2$Ntriazole), 36.2 (CH$_2$triazole), 34.5 (CH$_2$S), 34.1 (C-2$_{Hex}$), 33.0 (C-6), 31.3 (C-4$_{Hex}$), 28.2 (*C*Me$_3$), 24.5 (C-3$_{Hex}$), 22.2 (C-5$_{Hex}$), 13.9 (C-6$_{Hex}$).

ESIMS: *m/z* = 1637.8 [M + Na]$^+$, 1679.7 [M + Cu]$^+$. Anal. Calcd for $C_{74}H_{126}N_{12}O_{19}S_4$: C, 55.00; H, 7.86; N, 10.40; S, 7.94. Found: C, 54.71; H, 7.59; N, 10.09; S, 7.64.

6,6'-Di-[2-(4-(*N*'-(2-aminoethyl)thioureidomethyl)-1*H*-1,2,3-triazol-1-yl)ethylthio]-6,6'-dideoxy-2,3,4,2',3',4'-hexa-*O*-hexanoyl-α,α'-trehalose dihydrochloride (117). Treatment of **116** (101 mg, 0.06 mmol) with 1:1 TFA-DCM (2 mL) as described in general methods yielded **117**. Yield: 90 mg (quantitative). $[\alpha]_D$ = + 26.0 (*c* 1.0, MeOH); UV (MeOH): λ_{max} = 244 nm (ε_{mM} 7.7); IR: ν_{max} = 2929, 1750, 1152 cm^{-1}.

^{1}H NMR (300 MHz, CD$_3$OD): δ = 8.03 (s, 2 H, =CH), 5.49 (t, 2 H, $J_{2,3} = J_{3,4}$ = 9.4 Hz, H-3), 5.36 (d, 2 H, $J_{1,2}$ = 3.8 Hz, H-1), 5.12 (dd, 2 H, H-2), 5.10 (t, 2 H, $J_{4,5}$ = 9.8 Hz, H-4), 4.81 (t, 4 H, $J_{H,H}$ = 6.9 Hz, CH$_2$-triazole), 4.59 (t, 4 H, $J_{H,H}$ = 6.7 Hz, SCH$_2$C*H*$_2$N), 3.96 (m, 2 H, H-5), 3.88 (t, 4 H, $J_{H,H}$ = 5.9 Hz, C*H*$_2$CH$_2$NH$_2$), 3.20 (t, 4 H, C*H*$_2$NH$_2$), 3.11 (m, 4 H, CH$_2$S), 2.65 (m, 4 H, H-6a, H-6b), 2.42-2.21 (m, 12 H, H-2$_{Hex}$), 1.59 (m, 12 H, H-3$_{Hex}$), 1.31 (m, 24 H, H-4$_{Hex}$, H-5$_{Hex}$), 0.91 (m, 18 H, H-6$_{Hex}$).

^{13}C NMR (75.5 MHz, CD$_3$OD): δ = 185.6 (CS), 174.2-173.8 (CO ester), 145.8 (C-4 triazole), 125.2 (C-5 triazole), 92.2 (C-1), 72.8 (C-4), 72.4 (C-3), 71.4 (C-2), 71.2 (C-5), 51.5 (SCH$_2$*C*H$_2$N), 42.6 (CH$_2$-triazole), 41.0 (CH$_2$NH$_2$), 40.3 (*C*H$_2$CH$_2$NH$_2$), 35.01 (C-2$_{Hex}$), 34.5 (CH$_2$S), 34.0 (C-6), 32.5 (C-4$_{Hex}$) 25.6 (C-3$_{Hex}$), 23.4 (C-5$_{Hex}$), 14.3 (C-6$_{Hex}$).

ESIMS: *m/z* = 1415.6 [M + H]$^+$. Anal. Calcd for $C_{64}H_{110}N_2O_{15}S_4 \cdot 2$ HCl: C, 51.63; H, 7.58; N, 11.29; S, 8.61. Found: C, 51.46; H, 7.41; N, 10.97; S, 8.30.

Dendritic trehalose derivative 118. To a solution of **115** (182 mg, 0.12 mmol) and Et_3N (47 μL, 0.71 mmol), *tert*-butyl *N*-(2-isothiocyanoethyl)carbamate **168** (144 mg, 0.71 mmol) was added, and the mixture was stirred at rt, under Ar atmosphere, for 48 h. The mixture was washed with HCl 0.1 N (2 x 10 mL) and the organic phase was dried ($MgSO_4$), filtered and concentrated. The residue was purified by column chromatography (4:1 EtOAc-cyclohexane → 9:1 DCM-MeOH). Yield: 184 mg (80%). R_f = 0.51 (9:1 DCM-MeOH); $[\alpha]_D$ = + 26.6 (*c* 0.7, DMF); UV (DMF-H_2O 0.4% v/v): λ_{max} = 247 nm (ε_{mM} 21.9); IR: ν_{max} = 3310, 2955, 1751, 1688, 968 cm^{-1}.

^1H NMR (300 MHz, DMSO-d_6): δ = 8.05 (s, 2 H, =CH), 7.59, 7.36 (bs, 4 H, NHCS), 6.80 (s, 2 H, NHBoc), 5.35 (t, 2 H, $J_{2,3} = J_{3,4}$ = 9.5 Hz, H-3), 5.25 (d, 2 H, $J_{1,2}$ = 3.7 Hz, H-1), 5.07 (dd, 2 H, H-2), 5.02 (t, 2 H, $J_{4,5}$ = 9.5 Hz, H-4), 4.49 (bt, 4 H, $J_{H,H}$ = 6.4 Hz, CH_2N), 3.88 (m, 2 H, H-5), 3.78 (s, 2 H, CH_2-triazole), 3.60-3.50 (m, 16 H, CH_2CH_2NHCS, CH_2CH_2NHBoc), 3.04 (m, 12 H, CH_2S, CH_2NHBoc), 2.73-2.50 (m, 12 H, NCH_2CH_2NHCS, H-6a, H-6b), 2.42-2.10 (m, 12 H, H-2_{Hex}), 1.58 (m, 12 H, H-3_{Hex}), 1.39 (s, 36 H, CMe_3), 1.25 (m, 24 H, H-4_{Hex}, H-5_{Hex}), 0.85 (m, 18 H, H-6_{Hex}).

^{13}C NMR (75.5 MHz, DMSO-d_6): δ = 172.5, 172.0 (CO ester), 155.6 (CO carbamate), 141.2 (C-4 triazole), 124.5 (C-5 triazole), 90.5 (C-1), 78.7 (CMe_3), 70.7 (C-4), 70.5 (C-2), 69.3 (C-5), 69.0 (C-3), 52.0 (NCH_2CH_2NHCS), 49.0 (CH_2N-triazole), 47.5 (CH_2-triazole), 43.3 (NCH_2CH_2NHCS), 42.1 (CH_2CH_2NHBoc), 33.5 (C-2_{Hex}), 32.5 (CH_2S), 32.0 (C-6), 31.9 (C-4_{Hex}), 28.5 (CMe_3), 23.5 (C-3_{Hex}), 21.5 (C-5_{Hex}), 13.5 (C-6_{Hex}).

ESIMS: *m/z* = 2255.9 $[M + Cu]^+$. Anal. Calcd for $C_{98}H_{174}N_{20}O_{23}S_6$: C, 53.67; H, 8.00; N, 12.77; S, 8.77. Found: C, 53.49; H, 7.88; N, 12.54; S, 8.39.

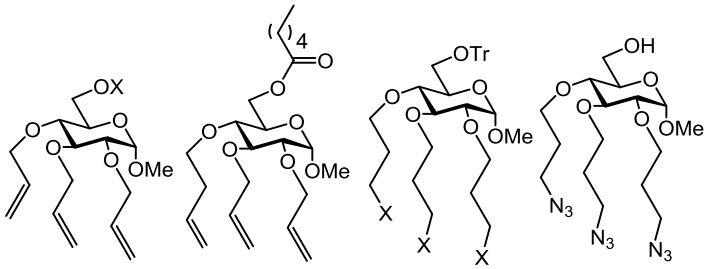
Dendritic trehalose derivative 119. Treatment of **118** (193 mg, 0.23 mmol) with 1:1 TFA-DCM (4 mL) as described in general methods yielded **119**. Yield: 167 mg (96%). $[\alpha]_D$ = + 28.6 (*c* 1.0, MeOH); UV (MeOH): λ_{max} = 244 nm (ε_{mM} 47.3); IR: ν_{max} = 2927, 2365, 1751, 1675, 1556, 721 cm^{-1}.

^{1}H NMR (300 MHz, CD_3OD): δ = 8.43 (s, 2 H, =CH), 5.50 (t, 2 H, $J_{2,3} = J_{3,4}$ = 9.5 Hz, H-3), 5.35 (d, 2 H, $J_{1,2}$ = 3.7 Hz, H-1), 5.12 (dd, 2 H, H-2), 5.07 (t, 2 H, $J_{4,5}$ = 9.5 Hz, H-4), 4.49 (bt, 4 H, $J_{H,H}$ = 6.2 Hz, CH_2N), 4.07 (bs, 8 H, NCH_2CH_2NHCS), 3.98 (m, 2 H, H-5), 3.87 (bt, 8 H, CH_2NH_2), 3.22 (bt, 8 H, NCH_2CH_2NHCS), 2.67 (m, 4 H, H-6a, H-6b), 2.32 (m, 12 H, H-2_{Hex}), 1.59 (m, 12 H, H-3_{Hex}), 1.32 (m, 24 H, H-4_{Hex}, H-5_{Hex}), 0.91 (m, 18 H, H-6_{Hex}).

^{13}C NMR (75.5 MHz, CD_3OD): δ = 174.8, 173.8 (CO), 143.4 (C4 triazole), 126.1 (C5 triazole), 92.3 (C-1), 72.6 (C-4), 72.3 (C-2), 71.3 (C-5), 71.1 (C-3), 52.0 (CH_2NH_2), 51.2 (CH_2Ntriazole), 47.2 (CH_2 triazole), 38.2 ($CH_2CH_2NH_2$), 35.1 (C-2Hex), 34.9 (CH_2S), 34.0 (C-6), 32.5 (C-4Hex), 25.6 (C-3Hex), 23.1 (C-5Hex), 14.0 (C-6Hex).

ESIMS: *m/z* = 1793 $[M + H]^+$, 896.9 $[M + H]^{2+}$. Anal. Calcd for $C_{78}H_{142}N_{20}O_{15}S_6 \cdot 4$ HCl: C, 48.33; H, 7.59; N, 14.45; S, 9.93. Found: C, 47.97; H, 7.22; N, 14.11; S, 9.55.

Secondary-Position Polycationic amphiphilic derivatives of glucose



120, X = Tr
121, X = H

122

123, X = OH
124 X = OMs
125 X = N_3

126

Methyl 2,3,4-tri-*O*-allyl-6-*O*-trityl-α-D-glucopyranoside (120). To a solution of methyl 6-*O*-trityl-α-D-glucopyranoside **171** (290 mg, 0.66 mmol) in dry DMF (5 mL), NaH (60% suspension in mineral oil, 240 mg, 5.96 mmol) was added at 0 ºC and the mixture was stirred for 15 min. Then, allyl bromide (0.51 mL, 59.4 mmol) was dropwise added, and the reaction mixture was stirred overnight, under Ar atmosphere, at rt. MeOH (2 mL) was then added, solvents were evaporated and the residue was diluted with DCM

(10 mL) and washed with water (2 x 10 mL). The organic layer was dried ($MgSO_4$), evaporated and purified by column chromatography (1:20 → 1:10 EtOAc-cyclohexane). Yield: 330 mg (93%). R_f = 0.45 (1:6 EtOAc-cyclohexane); $[\alpha]_D$ = +65.4 (*c* 1.0, DCM); IR: ν_{max} = 1076, 907 cm^{-1}.

^{1}HNMR (300 MHz, $CDCl_3$): δ = 7.45-7.10 (m, 15 H, aromatics), 5.97, 5.40 (m, 3 H, =CH), 5.29-4.85 (m, 6 H, $=CH_2$), 4.77 (d, 1 H, $J_{1,2}$ = 3.6 Hz, H-1), 4.32-3.62 (m, 7 H, OCH_2, H-5), 3.61 (t, 1 H, $J_{2,3}$ = $J_{3,4}$ = 9.4 Hz, H-3), 3.41-3.30 (m, 3 H, H-2, H-4, H-6a), 3.36 (s, 3 H, OCH_3), 3.04 (dd, 2 H, $J_{5,6b}$ = 4.6 Hz, $J_{6a,6b}$ = 10.2 Hz, H-6b).

^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 144.0, 129.8, 128.8, 127.9 (Ph), 135.3, 135.0, 134.7 (=CH), 117.4, 116.8 , 116.7 ($=CH_2$), 98.1 (C-1), 86.3 (Ph_3C), 81.7 (C-3), 79.7 (C-2), 78.0 (C-4), 74.5, 73.8, 70.1 (OCH_2), 72.6 (C-5), 62.6 (C-6), 54.9 (OCH_3).

ESIMS: *m/z* = 579.3 $[M + Na]^+$. Anal. Calcd for $C_{35}H_{40}O_5$: C, 77.75; H, 7.46. Found: C, 77.90; H, 7.49.

Mehyl 2,3,4-tri-*O*-allyl-α-D-glucopyranoside (121). To a solution of **120** (315 mg, 0.58 mmol) in 1:1 MeOH-DCM (6 mL), PTSA (45 mg, 0.23 mmol) was added and the mixture was stirred at rt for 3 h. A saturated aqueous solution of $NaHCO_3$ (10 mL) was added and the organic layer was dried ($MgSO_4$), filtered, concentrated and purified by column chromatography of the residue (1:5 → 1:3 → 2:1 EtOAc-cyclohexane). Yield: 162 mg (89%). R_f = 0.23 (1:1 EtOAc-cyclohexane); $[\alpha]_D$ = +108.9 (*c* 1.0, DCM); IR: ν_{max} = 3371, 1120 cm^{-1}.

^{1}HNMR (300 MHz, CD_3OD): δ = 6.06-5.86 (m, 3 H, =CH), 5.36-5.10 (m, 6 H, $=CH_2$), 4.8 (d, 1 H, $J_{1,2}$ = 3.6 Hz, H-1), 4.38-4.11 (m, 6 H, OCH_2,), 3.78 (dd, 1 H, $J_{5,6a}$ = 2.2 Hz, $J_{6a,6b}$ = 11.8 Hz, H-6a), 3.66 (dd, 1 H, $J_{5,6b}$ = 4.4 Hz, H-6b), 3.65 (t, 1 H, $J_{2,3}$ = $J_{3,4}$ = 9.4 Hz, H-3), 3.51 (m, 1 H, H-5), 3.40 (s, 3 H, OCH_2), 3.31 (m, 1 H, H-4).

^{13}C NMR (75.5 MHz, CD_3OD): δ = 137.8, 137.5, 137.2 (=CH), 118.5, 117.7, 117.5 ($=CH_2$), 100.2 (C-1), 83.6 (C-3), 82.1 (C-2), 79.7 (C-4), 76.2, 75.8, 79.7 (OCH_2), 74.2 (C-5), 63.0 (C-6), 56.4 (OCH_3).

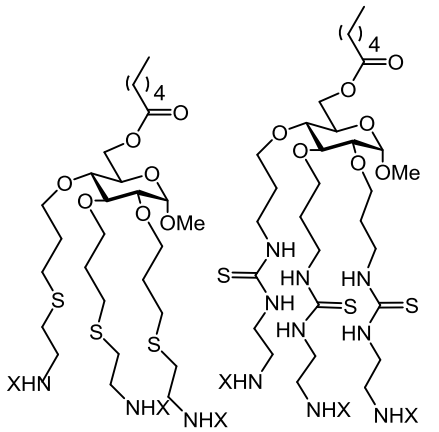
ESIMS: *m/z* = 337.1 $[M + Na]^+$. Anal. Calcd for $C_{16}H_{26}O_6$: C, 61.13; H, 8.34. Found: C, 61.04; H, 8.22.

Methyl 2,3,4-tri-*O*-allyl-6-*O*-hexanoyl-α-D-glucopyranoside (122). To a solution of **121** (140 mg, 0.44 mmol) and DMAP (109 mg, 0.89 mmol) in dry DMF (4 mL), hexanoic anhydride (0.26 mL, 1.11 mmol) was added dropwise and the mixture was stirred, under Ar atmosphere, for 3 h. Then, MeOH (3 mL) was added and the mixture was stirred for 1 h. The reaction mixture was poured onto ice-water (20 mL) and the aqueous solution was extracted with DCM (10 mL). The organic phase was then washed with 2 N H_2SO_4 (2 x 10 mL), H_2O (2 x 20 mL) and saturated aqueous $NaHCO_3$ (2 x 10 mL), dried ($MgSO_4$), filtered, concentrated and purified by column chromatography (1:10 EtOAc-petroleum ether). Yield: 138 mg (74%). R_f = 0.37 (1:3 EtOAc-cyclohexane); $[\alpha]_D$ = +87.9 (*c* 1.0, DCM); IR: ν_{max} = 1734, 1077 cm^{-1}.

^{1}H NMR (300 MHz, $CDCl_3$): δ = 6.06-5.77 (m, 3 H, =CH), 5.25-5.06 (m, 6 H, $=CH_2$), 4.73 (d, 2 H, $J_{1,2}$ = 3.6 Hz, H-1), 4.40-4.01 (m, 8 H, OCH_2, H-6a, H-6b), 3.74 (m, 1 H, H-5), 3.72 (t, 1 H, $J_{2,3} = J_{3,4}$ = 9.4 Hz, H-3), 3.39 (s, 3 H, OCH_3), 3.36 (dd, 1 H, H-2), 3.26 (m, 1 H, H-4), 2.33 (m, 2 H, $J_{H,H}$ = 7.7 Hz, H-2_{Hex}), 1.62 (m, 2 H, H-3_{Hex}), 1.29 (m, 4 H, H-4_{Hex}, H-5_{Hex}), 0.88 (m, 3 H, $J_{H,H}$ = 7.1 Hz, H-6_{Hex}).

^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 177.6 (CO ester), 135.2, 134.7, 134.5 (=CH), 117.7, 117.2, 116.7 ($=CH_2$), 98.1 (C-1), 81.4 (C-3), 79.4 (C-2), 77.5 (C-4), 74.3, 73.9, 72.6 (OCH_2), 68.6 (C-5), 62.9 (C-6), 55.1 (OCH_3), 34.1 (C-2_{Hex}), 31.3 (C-4_{Hex}), 24.6 (C-3_{Hex}), 22.3 (C-5_{Hex}), 13.9 (C-6_{Hex}).

ESIMS: *m/z* = 435.2 $[M + Na]^+$, 847.4 $[2M + Na]^+$. Anal. Calcd for $C_{68}H_{74}O_{11}$: C, 76.52; H, 6.99. Found: C, 76.33; H, 7.12.



129 X = Boc
130, X = H·HCl

131, X = Boc
132 X = H·HCl

Methyl 6-*O*-hexanoyl-2,3,4-tri-*O*-(3-(2-*N*-*tert*-butoxycarbonylaminoethylthio) propyl)-α-D-glucopyranoside (129). To a solution of **122** (83 mg, 0.19 mmol) in degassed MeOH (1 mL), 2-(*tert*-butoxycarbonylamino)ethanethiol (0.49 mL, 2.19 mmol) was irradiated at rt at 254 nm under magnetic stirring. Solvent was evaporated and the residue was purified by column chromatography (1:5 → 1:3 → 1:1 EtOAc-cyclohexane). Yield: 170 mg (95%). R_f = 0.28 (1:1 EtOAc-cyclohexane); $[\alpha]_D$ = +38.9 (*c* 1.0, DCM); IR: ν_{max} = 3364, 1707, 1508, 1165 cm^{-1}.

^{1}H NMR (300 MHz, $CDCl_3$): δ = 5.02, 4.97 (bs, 3 H, NHBoc), 5.75 (d, 1 H, $J_{1,2}$ = 3.5 Hz, H-1), 4.30 (dd, 2 H, $J_{6a,6b}$ = 12.1 Hz, $J_{5,6a}$ = 2.3 Hz, H-6a), 4.21 (dd, 2 H, $J_{5,6b}$ = 5.0 Hz, H-6b), 3.91-3.46 (m, 3 H, CH_2O, H-5, H-3), 3.54 (t, 1 H, $J_{2,3}$ = $J_{3,4}$ = 9.4 Hz, H-3), 3.39 (s, 3 H, OCH_3), 3.28 (m, 6 H, C*H*$_2$NHBoc), 3.23 (dd, 1 H, H-2), 3.13 (t, 2 H, $J_{4,5}$ = 9.4 Hz, H-4), 2.66 (m, 12 H, SC*H*$_2$CH$_2$NHBoc, CH_2S), 2.31 (t, 4 H, $J_{H,H}$ = 7.5 Hz, H-2$_{Hex}$), 1.92-1.70 (m, 12 H, C*H*$_2$CH$_2$O), 1.67 (m, 2 H, H-3$_{Hex}$), 1.42 (bs, 27 H, CMe_3), 1.36- 1.20 (m, 4 H, H-4$_{Hex}$, H-5$_{Hex}$), 0.88 (t, 6 H, $J_{H,H}$ = 6.8 Hz, H-6$_{Hex}$).

^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 173.6 (CO ester), 155.8 (CO carbamate), 97.6 (C-1), 81.4 (C-3), 80.6 (C-2), 79.3 (*C*Me$_3$), 78.1 (C-4), 71.8, 71.2, 68.7 (OCH_2), 69.5 (C-5), 62.9 (C-6), 55.1 (OCH_3), 39.8 (CH_2NHBoc), 34.2 (C-2$_{Hex}$), 32.2 (SC*H*$_2$CH$_2$NHBoc), 31.4

(C-4_{Hex}), 30.6, 30.1, 29.9 (C$H_2$$CH_2O$), 28.4 (C$Me_3$), 28.2 ($CH_2S$), 24.6 (C-$3_{Hex}$), 22.4 (C-$5_{Hex}$), 14.0 (C-$6_{Hex}$).

ESIMS: *m/z* = 966.5 $[M + Na]^+$. Anal. Calcd for $C_{43}H_{81}N_3O_{13}S_3$: C, 54.69; H, 8.65; N, 4.45; S, 10.19. Found: C, 54.73; H, 8.81; N, 4.33; S, 10.02.

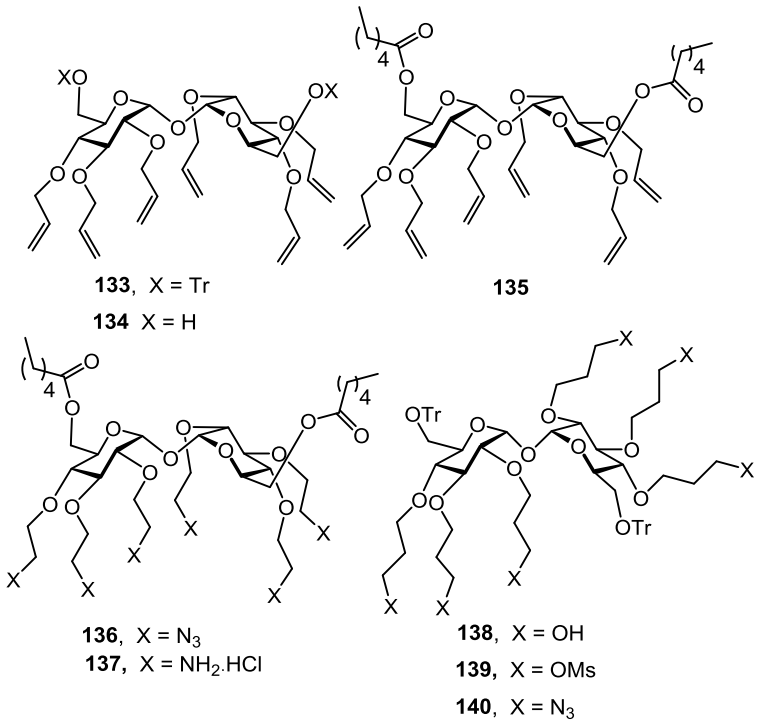
Methyl 2,3,4-tri-*O*-(3-(aminoethylthio)propyl)-6-*O*-hexanoyl-α-D-glucopyranoside hydrochloride (130). Treatment of compound **129** (117 mg, 0.13 mmol) with 1:1 TFA-DCM (2 mL), followed by freeze-drying from an aqueous HCl solution as indicated in general methods yielded **130**. Yield: 94 mg (99%). $[\alpha]_D$ = +38.6 (*c* 1.0, MeOH); IR: ν_{max} = 3335, 1682, 1022 cm^{-1}.

^{1}H NMR (300 MHz, CD_3OD): δ = 4.62 (d, 2 H, $J_{1,2}$ = 3.6 Hz, H-1), 4.36 (dd, 2 H, $J_{6a,6b}$ = 12.0 Hz, $J_{5,6a}$ = 2.1 Hz, H-6a), 4.18 (dd, 2 H, $J_{5,6b}$ = 5.2 Hz, H-6b), 3.96-3.56 (m, 7 H, CH_2O, H-5), 3.50 (t, 1 H, $J_{2,3}$ = $J_{3,4}$ = 9.4 Hz, H-3), 3.39 (s, 3 H, OCH_3), 3.27 (dd, 1 H, H-2), 3.22 (m, 1 H, H-4), 3.15 (m, 6 H, C$H_2$$NH_2$), 2.83 (m, 6 H, SC$H_2$$CH_2$$NH_2$), 2.69 (m, 6 H, CH_2S), 2.36 (t, 4 H, $J_{H,H}$ = 7.4 Hz, H-2_{Hex}), 1.87 (m, 6 H, C$H_2$$CH_2O$), 1.63 (m, 4 H, $J_{H,H}$ = 7.4 Hz H-3_{Hex}), 1.33 (m, 4 H, H-4_{Hex}, H-5_{Hex}), 0.92 (t, 3 H, $J_{H,H}$ = 6.8 Hz, H-6_{Hex}).

^{13}C NMR (75.5 MHz, CD_3OD): δ = 176.3 (CO ester), 99.8 (C-1), 83.5 (C-3), 82.8 (C-2), 80.6 (C-4), 73.6, 71.2 (OCH_2), 73.2 (C-5), 65.3 (C-6), 41.0 (CH_2NH_2), 36.1 (C-2_{Hex}), 32.5, 32.1, 32.0 (C$H_2$$CH_2O$), 30.8 (SC$H_2$$CH_2$NHBoc), 30.7 ($CH_2S$), 30.2 (C-$4_{Hex}$), 26.8 (C-$3_{Hex}$), 24.4 (C-$5_{Hex}$), 15.3 (C-$6_{Hex}$).

ESIMS: *m/z* = 644.3 $[M + H]^+$, 706.2 $[M + Na + K]^+$. Anal. Calcd for $C_{28}H_{57}N_3O_7S_3 \cdot 3$ HCl: C, 44.64; H, 8.03; N, 5.58; S, 12.77. Found: C, 44.30; H, 7.72; N, 5.21; S, 12.43.

Secondary-Position Polycationic amphiphilic derivatives of trehalose



2,3,4,2',3',4'-Hexa-*O*-allyl-6,6'-di-*O*-trityl-α,α'-trehalose (133). To a solution of 6,6'-di-*O*-trityl-α,α'-trehalose **173** (1 g, 1.2 mmol) in dry DMF (18 mL), NaH (60% suspension in mineral oil, 1.74 g, 43.3 mmol) was added at 0 ºC and the mixture was stirred for 15 min. Then, allyl bromide (3.68 mL, 43.3 mmol) was added dropwise, and the reaction mixture was stirred overnight, under Ar atmosphere, at rt. MeOH (5 mL) was then added, solvents were evaporated and the residue diluted in DCM (15 mL) and washed with H_2O (2 x 15 mL). The organic layer was dried ($MgSO_4$) and evaporated and the residue was purified by column chromatography (1:30 → 1:20 EtOAc-cyclohexane). Yield: 965 mg (76%). R_f = 0.33 (1:6 EtOAc-cyclohexane); $[\alpha]_D$ = +92.6 (*c* 1.0, DCM); IR: ν_{max} = 1265, 1074 cm^{-1}.

^1H NMR (300 MHz, $CDCl_3$): δ = 7.41-7.12 (m, 30 H, aromatics), 5.84, 5.54 (m, 6 H, =CH), 5.22 (d, 2 H, $J_{1,2}$ = 3.8 Hz, H-1), 5.18-4.83 (m, 12 H, $=CH_2$), 4.30-3.74 (m, 14 H, OCH_2, H-5), 3.60 (t, 2 H, $J_{2,3} = J_{3,4}$ = 9.2 Hz, H-3), 3.48 (t, 2 H, $J_{4,5}$ = 9.2 Hz, H-4), 3.39

(dd, 2 H, H-2), 3.34 (dd, 2 H, $J_{6a,6b}$ = 10.2 Hz, $J_{5,6a}$ = 1.8 Hz, H-6a), 3.03 (dd, 2 H, $J_{5,6b}$ = 3.7 Hz, H-6b).

^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 144.0, 128.8, 127.8, 126.9 (Ph), 135.4, 134.9, 134.8 (=CH), 116.6 (=CH_2), 94.1 (C-1), 86.3 (*C*Ph_3), 81.5 (C-3), 79.6 (C-2), 77.8 (C-4), 74.6 (OCH_2), 71.8 (C-5), 70.4 (OCH_2), 62.1 (C-6).

ESIMS: *m/z* = 1089.6 $[M + Na]^+$. Anal. Calcd for $C_{68}H_{74}O_{11}$: C, 76.52; H, 6.99. Found: C, 76.33; H, 7.12.

2,3,4,2',3',4'-Hexa-*O*-allyl-α,α'-trehalose (134). To a solution of **133** (860 mg, 0.8 mmol) in MeOH-DCM (1:1, 20 mL), PTSA (153 mg, 0.64 mmol) was added and the mixture was stirred at rt for 3 h. A saturated aqueous solution of $NaHCO_3$ (20 mL) was added and the organic layer was dried ($MgSO_4$), filtered and concentrated. The residue was purified by column chromatography (1:6 → 1:3 → 2:1 EtOAc-cyclohexane). Yield: 400 mg (85%). R_f = 0.35 (2:1 EtOAc-cyclohexane); $[\alpha]_D$ = +146.2 (*c* 1.0, DCM); IR: ν_{max} = 3403, 1068 cm^{-1}.

^{1}H NMR (300 MHz, CD_3OD): δ = 6.05, 5.83 (m, 6 H, =CH), 5.35-5.13 (m, 12 H, =CH_2), 5.19 (d, 2 H, $J_{1,2}$ = 3.6 Hz, H-1), 4.42-3.87 (m, 14 H, OCH_2, H-5), 3.75 (t, 2 H, $J_{2,3}$ = $J_{3,4}$ = 9.4 Hz, H-3), 3.67 (m, 4 H, H-6a, H-6b), 3.35 (m, 4 H, H-2, H-4).

^{13}C NMR (75.5 MHz, CD_3OD): δ = 136.9, 136.5, 136.2 (=CH), 117.0, 116.6, 116.4 (=CH_2), 94.3 (C-1), 82.2 (C-3), 80.7 (C-2), 78.4 (C-4), 75.1, 74.6, 72.8 (OCH_2), 72.9 (C-5), 61.9 (C-6).

ESIMS: *m/z* = 605.3 $[M + Na]^+$. Anal. Calcd for $C_{30}H_{46}O_{11}$: C, 61.84; H, 7.96. Found: C, 61.76; H, 7.89.

2,3,4,2',3',4'-Hexa-*O*-allyl-6,6'-di-*O*-hexanoyl-α,α'-trehalose (135). To a solution of **134** (370 mg, 0.64 mmol) and DMAP (465 mg, 3.81 mmol) in dry DMF (9 mL), hexanoic anhydride (1.1 mL, 5.08 mmol) was added dropwise and the mixture was stirred under Ar atmosphere for 3 h. MeOH (10 mL) was added and the mixture was stirred for 1

h, then the mixture was poured onto ice-water (30 mL) and the aqueous solution was extracted with DCM (15 mL). The organic phase was washed with 2 N H_2SO_4 (2 x 15 mL), H_2O (2 x 20 mL) and saturated aqueous $NaHCO_3$ (2 x 15 mL), dried ($MgSO_4$), filtered, concentrated and purified by column chromatography (1:10 EtOAc-petroleum ether). Yield: 386 mg (77%). R_f = 0.44 (1:5 EtOAc-cyclohexane); $[\alpha]_D$ = +119.6 (*c* 1.0, DCM); IR: ν_{max} = 1735, 1074 $cm^{-1.}$

^{1}H NMR (300 MHz, $CDCl_3$): δ = 6.08-5.75 (m, 6 H, =CH), 5.38-5.11 (m, 12 H, $=CH_2$), 5.08 (d, 2 H, $J_{1,2}$ = 3.7 Hz, H-1), 4.44-4.01 (m, 18 H, OCH_2, H-5, H-6a, H-6b), 3.72 (t, 2 H, $J_{2,3} = J_{3,4}$ = 9.5 Hz, H-3), 3.35 (dd, 2 H, H-2), 3.31 (t, 2 H, $J_{4,5}$ = 9.5 Hz, H-4), 2.30 (m, H-2_{Hex}), 1.61 (q, 4 H, $J_{H,H}$ = 7.2 Hz, H-3_{Hex}), 1.29 (m, 8 H, H-4_{Hex}, H-5_{Hex}), 0.88 (m, 6 H, H-6_{Hex}).

^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 173.5 (CO), 135.2, 134.7, 134.5 (=CH), 117.2, 117.1, 116.5 ($=CH_2$), 93.8 (C-1), 81.1 (C-3), 79.0 (C-2), 77.3 (C-4), 74.3, 74.0, 71.2 (OCH_2), 68.9 (C-5), 62.8 (C-6), 34.1 (C-2_{Hex}), 31.3 (C-4_{Hex}), 24.6 (C-3_{Hex}), 22.2 (C-5_{Hex}), 13.8 (C-6_{Hex}).

ESIMS: *m/z* = 801.4 $[M + Na]^+$. Anal. Calcd for $C_{42}H_{66}O_{13}$: C, 64.76; H, 8.54. Found: C, 64.80; H, 8.51.

2,2,3,3,4,4'-Hexa-*O*-(3-azidopropyl)-6,6'-di-*O*-hexanoyl-α,α'-trehalose (136). To a solution of **135** (110 mg, 0.13 mmol) and DMAP (88 mg, 0.71 mmol) in dry DMF (3 mL), hexanoic anhydride (0.14 mL, 0.59 mmol) was added dropwise and the mixture was stirred at rt, under Ar atmosphere for 3 h. MeOH (2 mL) was added and the mixture was stirred at rt for 1 h. The reaction mixture was poured onto ice-water (10 mL) and the aqueous solution was extracted with DCM (10 mL). The organic phase was then washed with 2 N H_2SO_4 (2 x 10 mL), H_2O (2 x 20 mL) and saturated aqueous $NaHCO_3$ (2 x 10 mL), dried ($MgSO_4$), filtered, concentrated and purified by column chromatography.

Yield: 140 mg (quantitative). R_f = 0.45 (1:5 EtOAc-cyclohexane); $[\alpha]_D$ = +80.2 (*c* 1.0, DCM); IR: ν_{max} = 2096, 1734 $cm^{-1.}$

^{1}H NMR (300 MHz, $CDCl_3$): δ = 5.09 (d, 2 H, $J_{1,2}$ = 3.6 Hz, H-1), 4.24 (m, 4 H, H-6a, H-6b) 3.92 (m, 2 H, H-5), 3.91-3.50 (14 H, CH_2O, H-3), 3.39 (m, 12 H, CH_2N_3), 3.26 (dd, 2 H, $J_{2,3}$ = 3.6 Hz, H-2), 3.19 (t, 2 H, $J_{3,4}$ = 3.6 Hz, H-4), 2.31 (t, 4 H, $J_{H,H}$ = 7.5 Hz, H-2_{Hex}), 1.94-1.74 (m, 12 H, CH_2), 1.62 (q, 2 H, $J_{H,H}$ = 7.5 Hz, H-3_{Hex}), 1.36-1.23 (m, 8 H, H-4_{Hex}, H-5_{Hex}), 0.88 (t, 6 H, $J_{H,H}$ = 7.0 Hz, H-6_{Hex}).

^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 173.4 (CO), 92.4 (C-1), 81.2 (C-3), 80.2 (C-2), 78.2 (C-4), 70.0, 69.7, 69.1 (CH_2O), 68.0 (C-5), 62.5 (C-6), 48.4, 48.3, 48.1 (CH_2N_3), 34.1 (C-4_{Hex}), 31.3 (C-5_{Hex}), 29.8, 29.6, 29.2 (CH_2), 24.6 (C-3_{Hex}), 22.3 (C-5_{Hex}), 13.9 (CH_3).

ESIMS: *m/z* = 1059.6 $[M + Na]^+$. Anal. Calcd for $C_{42}H_{72}N_{18}O_{13}$: C, 48.64; H, 7.00; N, 24.31. Found: C, 48.72; H, 7.11; N, 24.19.

2,2,3,3,4,4'-Hexa-*O*-(3-aminopropyl)-6,6'-di-*O*-hexanoyl-α,α'-trehalose hydrochloride (137). To a solution of **136** in THF (20 mL), TPP (425 mg, 1.62 mmol) was added and the mixture was stirred at rt for 15 min. Then NH_4OH (2 mL) was added and the solution was stirred overnight at 50 ºC. The mixture was concentrated and the resulting residue purified by column chromatography (EtOAc → 9:1 DCM-MeOH). The residue was dissolved in 10:1 H_2O-HCl 0.1 N and freeze-dried to yield the product as hydrochloride. Yield: 116 mg (98%). $[\alpha]_D$ = +71.5 (*c* 1.0, MeOH); IR: ν_{max} = 1730, 3337 cm^{-1}.

^{1}H NMR (300 MHz, CD_3OD): δ = 5.22 (s, 2 H, $J_{1,2}$ = 3.5 Hz, H-1), 4.35 (m, 4 H, H-6a, H-6b), 4.05- 3.66 (m, 16 H, CH_2O, H-3, H-5), 3.65 (t, 2 H, $J_{2,3}$ = $J_{3,4}$ = 9.4 Hz, H-3), 3.44 (dd, 2 H, H-2), 3.11 (m, 12 H, CH_2NH_2), 2.38 (t, 4 H, $J_{H,H}$ = 7.4 Hz, H-2_{Hex}), 2.04 (m, 8 H, H-4_{Hex}, H-5_{Hex}), 0.93 (t, 6 H, $J_{H,H}$ = 7.0 Hz, H-6_{Hex}).

^{13}C NMR (75.5 MHz, CD_3OD): 175.2 (CO), 94.0 (C-1), 82.5 (C-3), 81.2 (C-2), 79.6 (C-4), 71.9, 71.4, 70.7 (CH_2O), 69.1 (C-5), 63.8 (C-6), 39.0, 38.8, 38.5 (CH_2NH_2), 35.0 ($C\text{-}2_{Hex}$), 32.4 ($C\text{-}4_{Hex}$), 29.5, 29.4, 29.2 (CH_2), 25.8 ($C\text{-}3_{Hex}$), 23.4 ($C\text{-}5_{Hex}$), 13.4 (CH_3).

ESIMS: *m/z* = 881.6 $[M - Cl]^+$, 441.2 $[M - Cl + H]^{2+}$. Anal. Calcd for $C_{42}H_{84}N_6O_{13}\cdot 6$ HCl: C, 47.44; H, 8.44; N, 7.90. Found: C, 47.17; H, 8.21; N, 7.72.

2,3,4,2',3',4'-Hexa-*O*-(3-hydroxypropyl)-6,6'-di-*O*-trityl-α,α'-trehalose (138). To a solution of **133** (200 mg, 0.19 mmol) in anhydrous THF (6 mL), 9-BBN (0.5 m solution in THF, 6.75 mL) was added under Ar atmosphere and the reaction mixture was stirred at reflux temperature for 4 h. Then, excess of 9-BBN was quenched by dropwise addition of H_2O (3 mL) at 0 ºC, 3 M aqueous NaOH (7 mL) and 33% H_2O_2 (5 mL) were added at 0 ºC and the mixture was further stirred at rt overnight. The aqueous phase was saturated with solid K_2CO_3 and the organic phase was separated. The aqueous phase was further extracted with THF (2 x 10 mL). The organic phase was dried ($MgSO_4$), filtered and concentrated and the resulting residue was purified by column chromatography (9:1 EtOAc-EtOH → 45:5:3 EtOAc-EtOH-H_2O). Yield: 101 mg (69%). R_f = 0.24 (45:5:3 EtOAc-EtOH-H_2O); $[\alpha]_D$ = +53.9 (*c* 1, MeOH); IR: ν_{max} = 2953, 2928, 1068 cm^{-1}.

^{1}H NMR (300 MHz, CD_3OD): δ = 7.47-7.10 (m, 30 H, aromatics), 5.35 (d, 2 H, $J_{1,2}$ = 3.4 Hz, H-1), 3.84 (m, 6 H, OCHa), 4.75-3.26 (m, 28 H, OCHb, CH_2OH, H-3, H-2, H-4, H-6a), 3.23 (m, 2 H, H-5), 3.08 (dd, 2 H, $J_{6a,6b}$ = 10.2 Hz, $J_{5,6b}$ = 4.8 Hz, H-6b), 1.82-1.22 (m, 12 H, CH_2).

^{13}CNMR (75.5 MHz, CD_3OD): δ = 143.7, 128.8, 127.8, 127.1 (Ph), 91.2 (C-1), 86.4 (CPh_3), 81.1 (C-3), 80.0 (C-2), 78.7 (C-4), 71.2, 71.3, 70.9 (OCH_2), 69.2 (C-5), 62.1 (C-6), 60.9, 60.6, 60.2 (CH_2OH), 32.8, 32.4, 32.3 (CH_2).

ESIMS: *m/z* = 1197.4 $[M + Na]^+$. Anal. Calcd for $C_{68}H_{86}O_{17}$: C, 69.49; H, 7.27. Found: C, 69.25; H, 7.19.

2,3,4,2',3',4'-Hexa-*O*-(3-methanesulfonyloxypropyl)-6,6'-di-*O*-trityl-α,α'-trehalose (139). To a solution of **138** (300 mg, 0.255 mmol) and Et_3N (1.06 mL, 7.66 mmol) in dry DCM (9 mL), MsCl (0.35 mL, 4.59 mmol) was added at 0 ºC and the solution was stirred, under Ar atmosphere, for 30 min. The mixture was washed with a saturated aqueous solution of $NaHCO_3$ (2 x 10 mL) and HCl 0.1 N (1 x 10 mL). The organic layer was dried ($MgSO_4$), filtered and concentrated. The residue was purified by column chromatography (1:5 → 1:3 EtOAc-cyclohexane). Yield: 280 mg (98%). R_f = 0.66 (EtOAc); $[\alpha]_D$ = +49.1 (*c* 1, DCM); IR: ν_{max} = 1351, 1100 cm^{-1}.

^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.54- 7.15 (m, 30 H, aromatics), 5.36 (d, 2 H, $J_{1,2}$ = 3.6 Hz, H-1), 4.33 (t, 6 H, $CH_{2a}OMs$), 4.23-3.61 (m, 24 H, CH_2OMs, H-3, H-2, H-5, H-6a), 3.57 (t, 2 H, $J_{3,4}$ = $J_{4,5}$ = 9.3 Hz, H-4), 3.28-3.07 (m, 8 H, CH_2O, H-6b), 2.99, 2.93, 2.88 (s, 9 H, CH_3S), 2.10-1.75 (m, 12 H, CH_2).

^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 143.7, 128.8, 127.9, 127.2 (Ph), 92.4 (C-1), 86.5 (CPh_3), 81.3 (C-3), 80.3 (C-2), 78.6 (C-4), 70.8 (C-5), 68.9, 68.2, 67.5 (OCH_2), 67.3, 66.8, 66.7 (CH_2OMs), 62.2 (C-6), 37.3, 37.2 (CH_3SO_2), 30.4, 29.9 (CH_2).

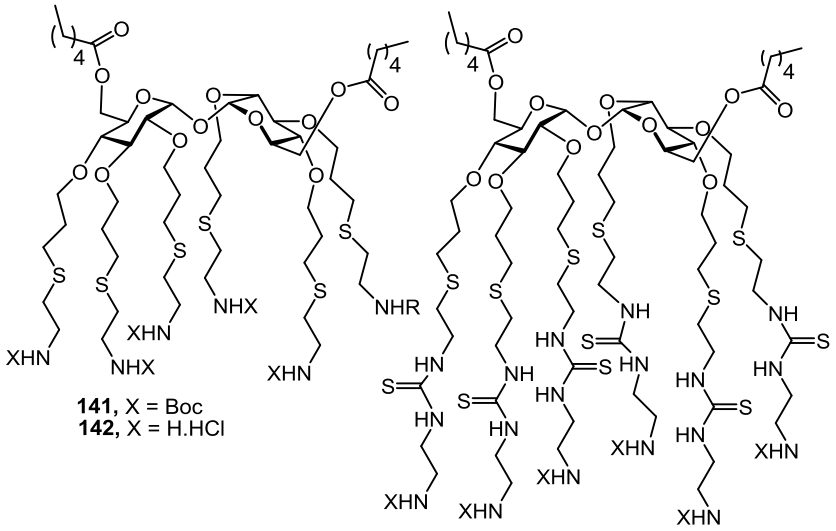
ESIMS: *m/z* = 1665.4 $[M + Na]^+$. Anal. Calcd for $C_{74}H_{98}O_{29}S_6$: C, 54.07; H, 6.01; S, 11.70. Found: C, 54.27; H, 6.19; S, 11.38.

2,3,4,2',3',4'-Hexa-*O*-(3-azidopropyl)-6,6'-di-*O*-trityl-α,α'-trehalose (140). To a solution of **139** (127 mg, 0.078 mmol) in dry DMF (2 mL), NaN_3 (93 mg, 1.42 mmol) was added and the suspension was stirred overnight, under Ar atmosphere. The solvent was evaporated, the residue diluted in DCM (10 mL) and washed with H_2O (2 x 10 mL). The organic layer was dried ($MgSO_4$), filtered and concentrated. Yield: 190 mg (quantitative). R_f = 0.57 (1:3 EtOAc-cyclohexane); $[\alpha]_D$ = +55.4 (*c* 1, DCM); IR: ν_{max} = 2096, 1092, 1008 cm^{-1}.

^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.55-7.25 (m, 30 H, aromatics), 5.39 (d, 2 H, $J_{1,2}$ = 3.5 Hz, H-1), 4.04-2.87 (m, 36 H, CH_2O, CH_2N_3, H-3, H-2, H-4, H-5, H-6a, H-6b), 1.98-1.47 (m, 12 H, CH_2).

^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 143.8, 128.8, 127.8, 127.1 (Ph), 92.6 (C-1), 86.5 (*C*Ph_3), 81.5 (C-3), 80.5 (C-2), 78.5 (C-4), 70.8 (C-5), 70.8, 70.0, 69.7 (OCH_2), 62.3 (C-6), 48.5, 48.3, 48.1 (CH_2N_3), 29.9, 29.5, 26.9 (CH_2).

ESIMS: *m/z* = 1374.4 $[M + Na]^+$. Anal. Calcd for $C_{68}H_{80}N_{18}O_{11}$: C, 61.62; H, 6.08; N, 19.02. Found: C, 61.80; H, 6.20; N, 18.89.



6,6'-Di-*O*-hexanoyl-2,3,4,2',3',4'-hexa-*O*-(3-(2-*N*-*tert*-butoxycarbonyl-aminoethylthio)propyl)-α,α'-trehalose (141). To a solution of **135** (100 mg, 0.128 mmol) in degassed MeOH (1 mL), 2-(Boc-amino)ethanethiol (0.68 mL, 3.85 mmol) was irradiated at rt at 254 nm under stirring. Solvent was evaporated and the residue purified by column chromatography (1:6 → 1:3 → 1:1 EtOAc-cyclohexane. Yield: 219 mg (93%). R_f = 0.58 (1:1 EtOAc-cyclohexane); $[\alpha]_D$ = +41.0 (*c* 1.0, DCM); IR: ν_{max} = 3361, 1699, 1508, 1163 cm^{-1}.

^{1}H NMR (300 MHz, $CDCl_3$): δ = 5.11 (bs, 6 H, NHBoc), 5.06 (d, 2 H, $J_{1,2}$ = 3.5 Hz, H-1), 4.26 (dd, 2 H, $J_{6a,6b}$ = 12.1 Hz, $J_{5,6a}$ = 4.3 Hz, H-6a), 4.18 (dd, 2 H, $J_{5,6b}$ = 2.1 Hz,H-6b), 4.00-3.46 (m, 16 H, CH_2O, H-5, H-3), 3.29 (m, 12 H, C*H*$_2$NHBoc), 3.23 (dd, 2 H, $J_{2,3}$ = 9.6 Hz, H-2), 3.18 (t, 2 H, $J_{4,5}$ = 9.6 Hz, H-4), 2.60 (m, 24 H, SC*H*$_2$CH$_2$NHBoc, CH_2S), 2.31 (t, 4 H, $J_{H,H}$ = 7.5 Hz, H-2$_{Hex}$), 1.94-1.72 (m, 12 H, C*H*$_2$CH$_2$O), 1.61 (m, 4 H, H-3$_{Hex}$), 1.42 (bs, 54 H, CMe_3), 1.34-1.20 (m, 8 H, H-4$_{Hex}$, H-5$_{Hex}$), 0.88 (t, 6 H, $J_{H,H}$ = 6.2 Hz, H-6$_{Hex}$).

^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 173.5 (CO ester), 155.8 (CO carbamate), 92.8 (C-1), 81.2 (C-3), 80.2 (C-2), 79.3 (*C*Me$_3$), 78.0 (C-4), 71.8, 71.4, 69.0 (OCH_2), 69.7 (C-5), 62.7 (C-6), 39.8 (CH_2NHBoc), 34.2 (C-2$_{Hex}$), 32.2 (SC*H*$_2$CH$_2$NHBoc), 31.3 (C-4$_{Hex}$), 30.6, 30.1, 30.0 (C*H*$_2$CH$_2$O), 28.4 (C*Me*$_3$), 28.1 (CH_2S), 24.6 (C-3$_{Hex}$), 22.3 (C-5$_{Hex}$), 14.1 (C-6$_{Hex}$).

ESIMS: *m/z* = 1843.8 $[M + Na]^+$, 943.3 $[2M + Na]^+$. Anal. Calcd for $C_{84}H_{156}N_6O_{25}S_6$: C, 54.76; H, 8.53; N, 4.56; S, 10.44. Found: C, 54.81; H, 8.34; N, 427; S, 10.25.

6,6'-Di-*O*-hexanoyl-2,3,4,2',3',4'-hexa-*O*-(3-(aminoethylthio)propyl)-α,α'-trehalose hydrochloride (142). Treatment of compound **141** (170 mg, 0.092 mmol) with 1:1 TFA-DCM (2 mL) as indicated in general methods yielded **142** as hydrochloride. Yield: 133 mg (99%). $[\alpha]_D$ = +59.8 (*c* 1.0, MeOH); IR: ν_{max} = 3368, 1676, 1020 cm^{-1}.

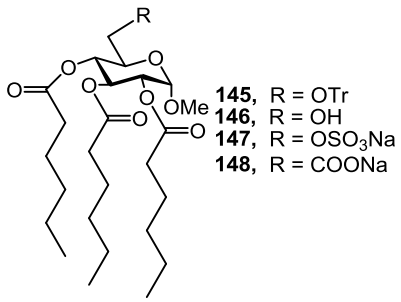
^{1}H NMR (300 MHz, CD_3OD): δ = 5.14 (d, 2 H, $J_{1,2}$ = 3.5 Hz, H-1), 4.33 (dd, 2 H, $J_{6a,6b}$ = 12.0 Hz, $J_{5,6a}$ = 2.2 Hz, H-6a), 4.22 (dd, 2 H, $J_{5,6b}$ = 4.5 Hz, H-6b), 3.97 (m, 2 H, H-5), 3.95-3.65 (m, 12 H, CH_2O), 3.60 (t, 2 H, $J_{2,3}$ = $J_{3,4}$ = 9.4 Hz, H-3), 3.32 (m, 2 H, H-2), 3.25 (t, 2 H, $J_{4,5}$ = 9.4 Hz, H-4), 3.16 (m, 12 H, C*H*$_2$NH$_2$), 2.83 (m, 12 H, SC*H*$_2$CH$_2$NH$_2$), 2.69 (m, 12 H, CH_2S), 2.36 (t, 4 H, $J_{H,H}$ = 7.2 Hz, H-2$_{Hex}$), 1.90 (q, 12 H, $J_{H,H}$ = 7.0 Hz, C*H*$_2$CH$_2$O), 1.63 (q, 4 H, $J_{H,H}$ = 7.2 Hz H-3$_{Hex}$), 1.34 (m, 8 H, H-4$_{Hex}$, H-5$_{Hex}$), 0.92 (t, 6 H, $J_{H,H}$ = 6.9 Hz, H-6$_{Hex}$).

^{13}C NMR (75.5 MHz, CD_3OD): δ = 175.3 (CO ester), 93.4 (C-1), 82.7 (C-3), 81.6 (C-2), 79.5 (C-4), 72.7, 72.3, 70.5 (OCH_2), 70.9 (C-5), 64.0 (C-6), 40.0 (CH_2NH_2), 35.1 (C-

2_{Hex}), 32.4 (C-4_{Hex}), 31.5, 31.2, 31.0 (C$H_2$$CH_2O$), 29.9 (SC$H_2$$CH_2$NHBoc), 29.2 ($CH_2S$), 25.8 (C-$3_{Hex}$), 22.4 (C-$5_{Hex}$), 14.3 (C-$6_{Hex}$).

ESIMS: *m/z* = 1241.6 $[M + H]^+$, 943.3 $[M + 2H]^{2+}$. Anal. Calcd for $C_{54}H_{108}N_6O_{13}S_6 \cdot 6$ HCl: C, 44.41; H, 7.87; N, 5.75; S, 13.17. Found: C, 44.11; H, 7.59; N, 5.48; S, 12.91.

Primary-position anionic amphiphilic derivatives of glucose.



Methyl 2,3,4-tri-*O*-hexanoyl-6-*O*-trityl-α–D-glucopyranoside (145). To a solution of methyl-6-*O*-trityl-α-D-glucopyranoside **171** (700 mg, 1.60 mmol) in dry DMF (12 mL) and DMAP (0.88 g, 7.2 mmol), hexanoic anhydride (2.2 mL, 9.6 mmol) was added dropwise at 0º C and the solution was stirred at rt, under Ar atmosphere, for 3 h. Then, MeOH (10 mL) was added and the mixture was stirred at rt for 2 h. The reaction mixture was poured onto ice-water (12 mL) and the aqueous solution was extracted with DCM (100 mL). The organic phase was then washed with H_2O (3 x 10 mL), dried ($MgSO_4$), filtered, concentrated and purified by column chromatography (1:40 → 1:30 → 1:20 EtOAc-petroleum ether)**.** Yield: 1.03 g (88%). R_f = 0.57 (1:6 EtOAc-cyclohexane); $[\alpha]_D$ = +65.8 (*c* 1.0, DCM); IR: ν_{max} = 2956, 2930, 1747, 1039 cm^{-1}.

^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.47-7.21 (m, 15 H, Ph), 5.49 (t, 1 H, $J_{2,3} = J_{3,4}$ = 9.6 Hz, H-3), 5.04 (t, 1 H, $J_{4,5}$ = 9.6 Hz, H-4), 5.03 (d, 1 H, $J_{1,2}$ = 3.6 Hz, H-1), 4.93 (dd, 1 H, H-2), 3.98 (m, 1 H, H-5), 3.50 (s, 3 H, OMe), 3.15 (m, 2 H, H-6a, H-6b), 2.39-1.80 (m, 6 H, $COCH_2$), 1.71-1.07 (m, 18 H, CH_2), 0.90 (m, 9 H, CH_3).

^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 173.1, 172.7, 172.0 (CO), 143.4, 128.7, 127.8, 127.0 (Ph), 96.6 (C-1), 86.6 (*C*Ph_3), 71.01 (C-3), 70.0 (C-2), 68.9 (C-4), 68.8 (C-5), 62.4 (C-6), 55.1 (OMe), 34.1, 33.8, 31.2, 24.6, 24.5, 24.2, 22.3, 22.2, 22.1 (CH_2), 13.8 (CH_3).

ESIMS: *m/z* = 753.5 $[M + Na]^+$. Anal. Calcd for $C_{44}H_{58}O_9$: C, 72.30; H, 8.00. Found: C, 72.48; H, 8.16.

Methyl 2,3,4-tri-*O*-hexanoyl-α–D-glucopyranoside (146). Trityl cleavage of the compound **145** (810 mg, 1.10 mmol) was achieved by treatment with PTSA (253 mg, 1.33 mmol) in 1:1 DCM-MeOH (12 mL) as indicated in general methods. The resulting residue was purified by column chromatography (1:10 → 1:6 → 1:3 EtOAc-cyclohexane). Yield: 430 mg (80%). R_f = 0.23 (1:6 EtOAc-cyclohexane); $[\alpha]_D$ = +87.8 (*c* 1.0, DCM); IR: ν_{max} = 2958, 2932, 1746, 1096 cm^{-1}.

^{1}H NMR (300 MHz, $CDCl_3$): δ = 5.56 (t, 1 H, $J_{2,3}$ = $J_{3,4}$ = 9.6 Hz, H-3), 4.95 (d, 1 H, $J_{1,2}$ = 3.7 Hz, H-1), 4.99 (d, 1 H, $J_{4,5}$ = 9.6 Hz, H-4), 4.85 (dd, 1 H, H-2), 3.75 (m, 1 H, H-5), 3.68 (dd, 1 H, $J_{6a,6b}$ = 13.0 Hz, $J_{5,6a}$ = 1.7 Hz, H-6a), 3.55 (dd, 1 H, $J_{5,6b}$ = 4.0 Hz, H-6b), 3.38 (s, 3 H, OMe), 2.33-2.20 (m, 6 H, $COCH_2$), 1.63-1.52 (m, 6 H, CH_2), 1.34-1.18 (m, 24 H, CH_2), 0.91-0.82 (m, 9 H, CH_3).

^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 173.5, 172.9, 172.6 (CO), 96.9 (C-1), 70.9 (C-3), 69.4 (C-2), 69.3 (C-4), 68.7 (C-5), 61.0 (C-6), 55.4 (OMe), 34.1, 34.0, 31.1, 24.0, 24.5, 22.2 (CH_2), 13.8 (CH_3).

ESIMS: *m/z* = 511.4 $[M + Na]^+$. Anal. Calcd for $C_{25}H_{44}O_9$: C, 61.45; H, 9.08. Found: C, 61.53; H, 9.19.

Methyl 2,3,4-tri-*O*-hexanoyl-6-sulfo-α-D-glucopyranoside sodium salt (147). Sulfur trioxide complex (130 mg, 0.82 mmol) was added to a solution of **146** (80 mg, 0.16 mmol) in dry pyridine (2 mL). After stirring, under Ar atmosphere, at 60 ºC for 15 min using MW radiation (20 W average power), the solvent was removed in vacuo. The resulting residue was purified by column chromatography on silica gel (30:1 DCM-

MeOH) and column chromatography on IRA-120 (sodium form). Yield: 98 mg (quantitative). R_f = 0.52 (6:1 DCM-MeOH); $[\alpha]_D$ = +116 (*c* 1.0, DCM); IR: ν_{max} = 2932, 1740, 731 cm^{-1}.

^1H NMR (300 MHz, $CDCl_3$): δ = 5.49 (t, 1 H, $J_{2,3} = J_{3,4}$ = 9.7 Hz, H-3), 5.13 (t, 1 H, $J_{4,5}$ = 9.7 Hz, H-4), 4.99 (bs, 1 H, H-1), 4.09 (dd, 1 H, $J_{1,2}$ = 1.7 Hz, H-2), 4.15 (m, 2 H, H-5, H-6a), 4.00 (d, 1 H, $J_{5,6a}$ = 1.7 Hz, H-6b), 3.41 (s, 3 H, OMe), 2.43-2.11 (m, 6 H, $COCH_2$), 1.66-1.43 (m, 12 H, CH_2), 1.39-1.15 (bs, 24 H, CH_2), 0.89 (m, 12 H, CH_3).

^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 173.6, 173.0, 172.5 (CO), 96.9 (C-1), 70.6 (C-3), 69.7 (C-2), 68.3 (C-4), 67.4 (C-5), 66.1 (C-6), 55.6 (OMe), 34.1 34.0, 31.2, 31.1, 24.5, 24.5, 24.4, 22.2 (CH_2), 13.8 (CH_3).

ESIMS: *m/z* = 567.2 $[M - H]^-$. Anal. Calcd for $C_{25}H_{43}NaO_{12}S$: C, 50.84; H, 7.34; S, 5.43. Found: C, 50.51; H, 6.99; N, 5.10.

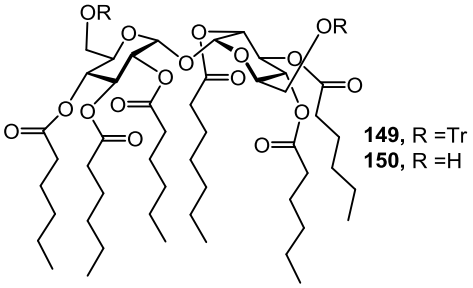
Methyl 2,3,4-tri-*O*-hexanoyl-α-D-glucopyranosyluronate sodium salt (148). To a solution of **146** (80 mg, 0.163 mmol) in CH_2Cl_2 (5 mL) Dess-Martin periodinane reagent (346 mg, 0.815 mmol) was added and the mixture was stirred overnight at rt. Et_2O (5 mL) and a saturated aqueous solution of $NaHCO_3$ containing 0.6 g of $Na_2S_2O_3$ (7.5 mL) was added and the mixture was stirred for 16 h. The mixture was diluted with Et_2O (10 mL) and washed a saturated aqueous solution of $NaHCO_3$ (2 x 10 mL) and H_2O. The organic layer was dried ($MgSO_4$), filtered and concentrated. To the resulting residue was added ^tBuOH (3 mL), a solution of methyl-but-2-ene (1 mL) in THF (1.5 mL) and a solution containing $NaClO_2$ (126 mg, 1.39 mmol) and NaH_2PO_4 (63 mg, 0.52 mmol) in H_2O, and the mixture was stirred overnight at rt. Aqueous HCl (1 N, 100 mL, to pH = 6) and EtOAc (10 mL) were added, the organic layer was separated, dried ($MgSO_4$) and concentrated. The residue was purified by column chromatography (50:1 DCM-MeOH, charged with 1% HCOOH). Yield: 60 mg (73%). R_f = 0.20 (EtOAc); $[\alpha]_D$ = +85.5 (*c* 1.0, DCM); IR: ν_{max} = 1740, 731 cm^{-1}.

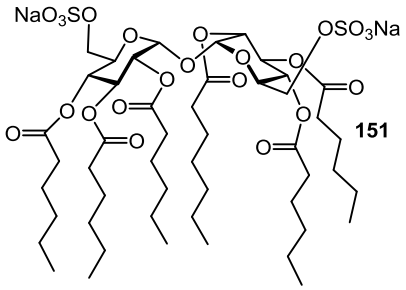
^{1}H NMR (300 MHz, $CDCl_3$): δ = 5.55 (t, 1 H, $J_{2,3}$ = $J_{3,4}$ = 9.5 Hz, H-3), 5.21 (t, 1 H, $J_{4,5}$ = 9.7 Hz, H-4), 5.05 (d, 1 H, $J_{1,2}$ = 3.4 Hz, H-1), 4.88 (dd, 1 H, H-2), 4.32 (d, 1 H, H-5), 3.43 (s, 3 H, OMe), 2.35-2.18 (m, 6 H, $COCH_2$), 1.64-1.47 (m, 12 H, CH_2), 1.39-1.15 (bs, 24 H, CH_2), 0.89 (m, 12 H, CH_3).

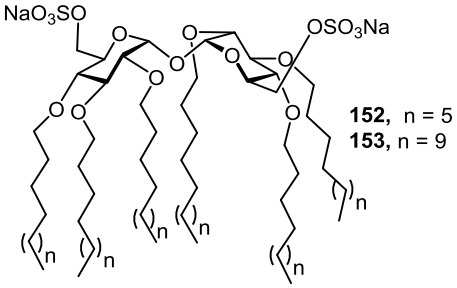
^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 173.0, 172.6, 171.5 (CO), 97.1 (C-1), 70.3 (C-3), 69.1 (C-2), 68.9 (C-4), 67.7 (C-5), 56.0 (OMe), 34.1, 34.0, 33.9, 31.2, 31.1, 24.6, 24.5, 24.3, 22.3 (CH_2), 13.8 (CH_3).

ESIMS: *m/z* = 501.2 $[M - H]^-$. Anal. Calcd for $C_{25}H_{42}O_{10}$: C, 59.74; H, 8.42; O. Found: C, 63.69; H, 10.21.

Primary-position anionic amphiphilic derivatives of trehalose.







2,3,4,2',3',4'-Hexa-*O*-hexanoyl-6,6'-disulfate-α,α'-trehalose disodium salt (151). Sufur trioxide complex (355 mg, 2.23 mmol) was added to a solution of diol **50** (80 mg, 0.086 mmol) in dry pyridine (2 mL). After stirring, under Ar atmosphere, at 60 ºC for 15

min using MW (20 w average power), the solvent was removed in vacuo. The resulting residue was purified by column chromatography on silica gel (9:1 DCM-MeOH) and successive column chromatography on IRA-120 (sodium form). Yield: 98 mg (quantitative). R_f = 0.41 (9:1 DCM-MeOH); $[\alpha]_D$ = +40 (*c* 1.0, DMF); IR: ν_{max} = 3486, 2953, 1754, 1002 cm^{-1}.

^1H NMR (300 MHz, CD_3OD): δ = 5.56 (t, 2 H, $J_{2,3} = J_{3,4}$ = 9.6 Hz, H-3), 5.38 (d, 2 H, $J_{1,2}$ = 3.7 Hz, H-1), 5.13 (dd, 2 H, H-2), 5.11 (d, 2 H, $J_{4,5}$ = 9.6 Hz, H-4), 4.19 (dt, 2 H, $J_{5,6a} = J_{5,6b}$ = 4.1 Hz, H-5), 4.02 (d, 4 H, H-6a, H-6b), 2.46-2.25 (m, 12 H, $COCH_2$), 1.68-1.54 (m, 12 H, CH_2), 1.38-1.29 (bs, 24 H, CH_2), 0.93 (t, 12 H, $J_{H,H}$ = 6.4 Hz, CH_3).

^{13}C NMR (75.5 MHz, CD_3OD): δ = 174.2, 174.1, 173.8 (CO), 92.9 (C-1), 71.7 (C-3), 70.9 (C-2), 70.0 (C-4), 67.1 (C-5), 55.1 (C-6), 35.0, 34.9, 32.5, 32.4, 26.5, 26.5, 23.4, 23.3 (CH_2), 14.3, 14.2 (CH_3).

ESIMS: *m/z* = 1111.5 $[M - Na]^-$. Anal. Calcd for $C_{25}H_{43}NaO_{12}S$: C, 50.84; H, 7.34; S, 5.43 Found: C, 50.49; H, 7.20; N, 5.40.

2,3,4,2',3',4'-Hexa-*O*-hexyl-6,6'-disulfo-α,α'-trehalose disodium salt (152). Sulfur trioxide complex (355 mg, 2.23 mmol) was added to a solution of **74** (189 mg, 0.223 mmol) in DMF (13 mL). After stirring, under Ar atmosphere, at 50 ºC for 2 h the solvent was removed and the resulting residue was purified by silica gel column chromatography (9:1 → 5:1 DCM-MeOH) and column chromatography on IRA-120 (sodium form). Yield: 262 mg (quantitative). R_f = 0.15 (5:1 DCM-MeOH); $[\alpha]_D$ = +56 (*c* 1.0, DMF); IR: ν_{max} = 3494, 2927, 2853, 1229, 1092 cm^{-1}.

^1H NMR (400 MHz, DMSO-d_6, 323 K): δ = 5.01 (d, 2 H, $J_{1,2}$ = 3.5 Hz, H-1), 3.90 (dd, 2 H, $J_{6a,6b}$ = 10.5 Hz, $J_{5,6a}$ = 3.2 Hz, H-6a), 3.79 (m, 4 H, H-5, H-6b), 3.73-3.39 (m, 12 H, OCH_2), 3.39 (t, 2 H, $J_{2,3} = J_{3,4}$ = 9.4 Hz, H-3), 3.15 (t, 2 H, $J_{4,5}$ = 9.4 Hz, H-4), 3.14 (dd, 2 H, H-2), 1.54-1.41 (m, 12 H, CH_2), 1.34-1.25 (m, 36 H, CH_2), 0.86 (m, 18 H, CH_3).

^{13}C NMR (100.6 MHz, DMSO-d_6, 323 K): δ = 91.8 (C-1), 80.5 (C-3), 79.4 (C-2), 76.8 (C-4), 72.0, 71.5, 70.0 (OCH_2), 69.3 (C-5), 63.8 (C-6), 31.0, 29.9, 29.5, 28.5, 25.1 (CH_2), 13.6 (CH_3).

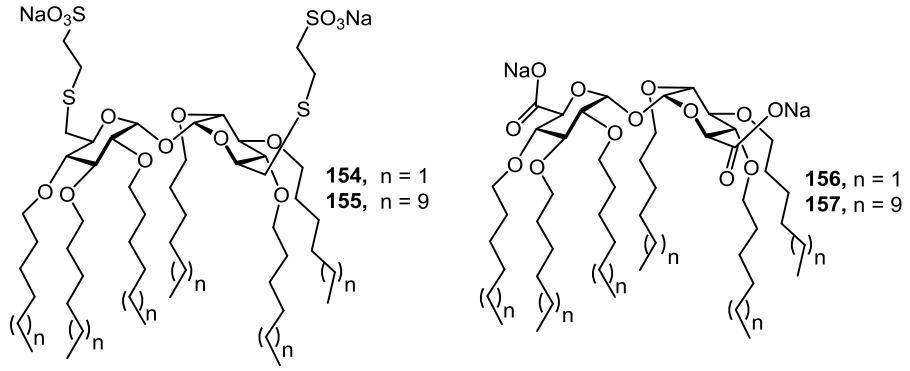
ESIMS: *m/z* = 1027.5 $[M + Na]^-$. Anal. Calcd for $C_{48}H_{92}Na_2O_{17}S_2$: C, 54.84; H, 8.82; S, 6.10. Found: C, 54.56; H, 8.62; N, 5.77.

2,3,4,2',3',4'-Hexa-*O*-tetradecyl-6,6'-disulfo-α,α'-trehalose disodium salt (153). Sufur trioxide complex (112 mg, 0.704 mmol) was added to a solution of diol **75** (107 mg, 0.070 mmol) in dry DMF (4 mL). After stirring, under Ar atmosphere, at 70 ºC for 3 h, and the solvent was removed and the resulting residue was purified by column chromatography on silica gel (9:1 → 5:1 DCM-MeOH) and column chromatography on IRA-120 (sodium form). Yield: 85 mg (70%). R_f = 0.33 (5:1 DCM-MeOH); $[\alpha]_D$ = +37 (*c* 1.0, DMF); IR: ν_{max} = 2922, 2848, 1219, 1092 cm^{-1}.

^{1}H NMR (400 MHz, DMSO-d_6, 353 K): δ = 5.01 (d, 2 H, $J_{1,2}$ = 3.4 Hz, H-1), 3.90 (dd, 2 H, $J_{6a,6b}$ = 10.9 Hz, $J_{5,6a}$ = 3.9 Hz, H-6a), 3.82 (m, 4 H, H-5, H-6b), 3.74-3.40 (m, 12 H, OCH_2), 3.47 (t, 2 H, $J_{2,3} = J_{3,4}$ = 9.2 Hz, H-3), 3.14 (t, 2 H, $J_{4,5}$ = 9.2 Hz, H-4), 3.13 (dd, 2 H, H-2), 1.55-1.45 (m, 12 H, CH_2), 1.29-1.26 (m, 132 H, CH_2), 0.86 (t, 18 H, $J_{H,H}$ = 7.1 Hz, CH_3).

^{13}C NMR (100.6 MHz, DMSO-d_6, 353 K): δ = 90.9 (C-1), 80.2 (C-3), 79.4 (C-2), 76.9 (C-4), 71.8, 71.0, 69.8 (OCH_2), 69.3 (C-5), 63.9 (C-6), 30.8, 29.7, 29.6, 28.6, 28.2, 25.4, 25.3, 25.2, 21.5 (CH_2), 13.2 (CH_3).

ESIMS: *m/z* = 838.6 $[M]^{2-}$, 1700.3 $[M + Na]^-$. Anal. Calcd for $C_{96}H_{188}Na_2O_{17}S_2$: C, 66.86; H, 10.99; S, 3.72. Found: C, 66.49; H, 10.68; N, 3.35.



6,6'-Di-(2-sulfonatoethylthio)-2,3,4,2',3',4'-hexa-*O*-hexyl-α,α'-trehalose disodium salt (154). To a solution of **76** (122 mg, 0.114 mmol) in dry DMF (7 mL), 1 M NaOMe (320 μL, 0.32 mmol) and sodium 2-mercaptoehanesulfonate (53 mg, 0.32 mmol) were added, under Ar atmosphere, and the reaction mixture was stirred at 70 ºC overnight. A 50% excess of reagent was additionally added to complete the reaction. The reaction mixture was concentrated and the residue was purified by column chromatography (1:5 EtOAc-cyclohexane → 45:5:3 EtOAc-EtOH-H_2O → 5:1 DCM-MeOH). Yield: 128 mg (98%). R_f = 0.14 (5:1 DCM-MeOH); $[\alpha]_D$ = +40 (*c* 1.0, DMF); IR: ν_{max} = 3414, 2951, 2918, 2859, 1618, 1097 cm^{-1}.

^{1}H NMR (300 MHz, DMSO-d_6): δ = 5.05 (d, 2 H, $J_{1,2}$ = 3.4 Hz, H-1), 3.81 (ddd, 2 H, $J_{4,5}$ = 9.3 Hz, $J_{5,6b}$ = 7.6 Hz, $J_{5,6a}$ = 2.3 Hz, H-5), 3.74-3.37 (m, 14 H, OCH_2, H-3), 3.23 (dd, 2 H, $J_{2,3}$ = 9.3 Hz, H-2), 3.05 (t, 2 H, $J_{3,4}$ = 9.3 Hz, H-4), 2.79 (dd, 2 H, $J_{6a,6b}$ = 14.4 Hz, H-6a), 2.74-2.58 (m, 10 H, H-6b, CH_2S, CH_2SO_3), 1.52-1.43 (m, 12 H, CH_2), 1.32-1.26 (m, 36 H, CH_2), 0.89 (t, 18 H, $J_{H,H}$ = 6.8 Hz, CH_3).

^{13}C NMR (75.5 MHz, DMSO-d_6): δ = 90.4 (C-1), 80.5 (C-3), 80.1 (C-2), 79.6 (C-4), 72.3, 70.2, 70.7 (OCH_2), 70.5 (C-5), 51.9 (CH_2SO_3), 33.4 (C-6), 31.3 (CH_2S), 31.1, 30.0, 29.9, 29.7, 28.5, 25.3, 25.2, 22.1, 22.0 (CH_2), 13.8 (CH_3).

ESIMS: *m/z* = 1115.8 $[M + Na]^-$. Anal. Calcd for $C_{52}H_{100}Na_2O_{15}S_4$: C, 54.81; H, 8.84; S, 11.26. Found: C, 54.49; H, 8.57; N, 10.82.

6,6'-Di-(2-sulfonatoethylthio)-2,3,4,2',3',4'-hexa-*O*-tetradecyl-α,α'-trehalose disodium salt (155). To a solution of **77** (150 mg, 0.086 mmol) in dry DMF (5 mL), 1 M NaOMe (241 μL, 0.241 mmol) and sodium 2-mercaptoehanesulfonate (40 mg, 0.241 mmol) were added, under Ar atmosphere, and the reaction mixture was stirred at 70 ºC overnight. A 50% excess of reagent was additionally added to complete the reaction. The reaction mixture was concentrated and the residue was purified by column chromatography (1:5 EtOAc-cyclohexane → 45:5:3 EtOAc-EtOH-H_2O → 5:1 DCM-MeOH). Yield: 97 mg (62%). R_f = 0.27 (5:1 DCM-MeOH); $[\alpha]_D$ = +11 (*c* 1.0, DMF); IR: ν_{max} = 2921, 2852, 1098, 1047 cm^{-1}.

^1H NMR (500 MHz, $CDCl_3$, 343 K): δ = 5.09 (bs, 2 H, H-1), 3.86 (ddd, 2 H, $J_{4,5}$ = 9.4 Hz, $J_{5,6b}$ = 7.2 Hz, $J_{5,6a}$ = 2.5 Hz, H-5), 3.74-3.58 (m, 8 H, OCH_2), 3.49 (m, 6 H, OCH_2, H-3), 3.17 (dd, 2 H, $J_{2,3}$ = 9.4 Hz, $J_{1,2}$ = 3.4 Hz H-2), 3.06 (t, 2 H, $J_{3,4}$ = 9.3 Hz, H-4), 2.88-2.74 (m, 8 H, $J_{6a,6b}$ = 14.4 Hz, H-6a, H6b, CH_2SO_3), 2.63 (m, 4 H, CH_2S), 1.55-1.48 (m, 12 H, CH_2), 1.25 (bs, 132 H, CH_2), 0.86 (t, 18 H, $J_{H,H}$ = 6.6 Hz, CH_3).

^{13}C NMR (100.6 MHz, $CDCl_3$, 323 K): δ = 92.4 (C-1), 83.3 (C-3), 82.8 (C-2), 82.4 (C-4), 74.8, 74.6 (OCH_2), 73.2 (C-5), 54.5 (CH_2SO_3), 35.7 (C-6), 33.4, 32,5, 32.4, 31.3, 31.2, 30.9 (CH_2), 30.5 (CH_2S), 28.2, 28.0, 24.2 (CH_2), 15.5 (CH_3).

ESIMS: *m/z* = 883.0 $[M]^{2-}$, 1788.2 $[M + Na]^-$. Anal. Calcd for $C_{100}H_{196}Na_2O_{15}S_4$: C, 66.25; H, 10.90; S, 7.08. Found: C, 66.09; H, 10.70; N, 6.84.

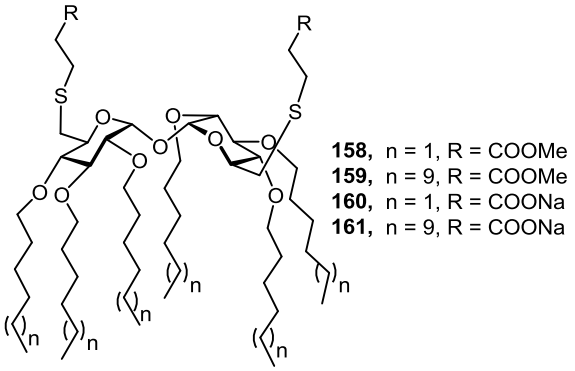
2,3,4,2',3',4'-Hexa-*O*-hexyl-α,α'-trehalosyluronate disodium salt (156). An aqueous solution of NaBr (1 m, 130 μL), TBAB (1 m, 260 μL), TEMPO (11 mg, 0.07 mmol), and a saturated aqueous solution of $NaHCO_3$ (650 μL) were added to a solution of **74** (100 mg, 0.118 mmol) in DCM (3.5 mL) and H_2O (600 μL) in ice-water bath. The resulted mixture was treated with NaOCl (750 μL) and stirred for 1 h as the temperature increased from 0 ºC to rt. The reaction media was neutralized with HCl (1 N, 400 μL) to pH 6-7. Then ^{t}BuOH (3.6 mL), 2-methylbut-2-ene (1.5 mL) and a solution of NaOCl (262 mg) and NaH_2PO_4 (210 mg) in H_2O (1.05 mL) were added and the mixture was stirred at

rt for 2 h, diluted with saturated aqueous solution of NaH_2PO_4 and extracted with DCM (3 x 10 mL), dried ($MgSO_4$) and concentrated. The residue was purified by column chromatography (30:1 → 20:1 DCM-MeOH charged with 1% HCOOH). Yield: 90 mg (79%). R_f = 0.7 (9:1 DCM-MeOH charged with 1% HCOOH); $[\alpha]_D$ = +74.6 (*c* 1.0, DCM); IR: ν_{max} = 2929, 2858, 1726, 1097 cm^{-1}.

^{1}H NMR (300 MHz, $CDCl_3$): δ = 5.15 (d, 2 H, $J_{1,2}$ = 3.4 Hz, H-1), 4.47 (d, 2 H, $J_{4,5}$= 10.0 Hz, H-5), 3.89-3.44 (m, 14 H, H-3, OCH_2), 3.41 (t, 2 H, $J_{3,4}$ = 10.0 Hz, H-4), 3.29 (dd, 2 H, $J_{2,3}$ = 10.0 Hz, H-2), 1.65-1.45 (m, 12 H, CH_2), 1.40-1.20 (bs, 36 H, CH_2), 0.87 (t, 18 H, $J_{H,H}$ = 7.0 Hz, CH_3).

^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 173.7 (CO), 94.5 (C-1), 80.9 (C-5), 79.7 (C-3), 79.6 (C-2), 73.9 (C-4), 73.7, 72.0, 69.8 (OCH_2), 31.9, 31.8, 30.6, 30.2, 26.3, 25.8, 22.8 (CH_2), 14.2 (CH_3).

ESIMS: *m/z* = 873.4 $[M]^-$. Anal. Calcd for $C_{48}H_{90}O_{13}$: C, 65.87; H, 10.37. Found: C, 65.51; H, 10.06.



158, n = 1, R = COOMe
159, n = 9, R = COOMe
160, n = 1, R = COONa
161, n = 9, R = COONa

6,6'-Di-(2-methoxycarbonylethylthio)-2,3,4,2',3',4'-hexa-*O*-hexyl-α,α'-trehalose (158). To a solution of **76** (200 mg, 0.187 mmol) in dry DMF (4 mL), Cs_2CO_3 (156 mg, 0.487 mmol) and methyl 2-mercaptopropionate (58 μL, 0.487 mmol) were added, under Ar atmosphere, and the reaction mixture was stirred at 70 ºC overnight. The reaction mixture was concentrated and the residue was purified by column chromatography (1:20

→ 1:15 → 1:6 EtOAc-cyclohexane). Yield: 150 mg (76%). R_f = 0.43 (1:6 EtOAc-cyclohexane); $[\alpha]_D$ = +76 (*c* 1.0, DCM); IR: ν_{max} = 2930, 2858, 1735, 1095 cm^{-1}.

^{1}H NMR (300 MHz, $CDCl_3$): δ = 5.11 (d, 2 H, $J_{1,2}$ = 3.6 Hz, H-1), 4.01 (ddd, 2 H, $J_{4,5}$ = 9.3 Hz, $J_{5,6b}$ = 6.3 Hz, $J_{5,6a}$ = 2.6 Hz, H-5), 3.85-3.77 (m, 4 H, OCH_2), 3.70-3.43 (m, 10 H, OCH_2, H-3), 3.68 (s, 6 H, OCH_3), 3.22 (dd, 2 H, $J_{2,3}$ = 9.3 Hz, H-2), 3.16 (t, 2 H, $J_{3,4}$ = 9.3 Hz, H-4), 2.85 (t, 4 H, $J_{H,H}$ = 7.4 Hz, SCH_2), 2.83 (dd, 2 H, $J_{6a,6b}$ = 13.5 Hz, H-6a), 2.72 (dd, 2 H, H-6b), 2.59 (t, 4 H, $J_{H,H}$ = 6.4 Hz, CH_2COO), 1.62-1.49 (m, 12 H, CH_2), 1.39-1.25 (m, 36 H, CH_2), 0.91-0.85 (m, 18 H, CH_3).

^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 172.4 (CO), 92.2 (C-1), 81.0 (C-3), 80.5 (C-2), 80.4 (C-4), 72.4, 73.2, 71.7 (OCH_2), 71.2 (C-5), 51.7 (OCH_3), 34.7 (C-6), 34.1 (CH_2S), 31.8 (CH_2COO), 31.7, 30.6, 30.4, 28.5, 25.9, 25.8, 22.6 (CH_2), 14.0 (CH_3).

ESIMS: *m/z* = 1747.3 $[M + Na]^-$. Anal. Calcd for $C_{56}H_{106}O_{13}S_2$: C, 63.96; H, 10.16; S, 6.10. Found: C, 64.11; H, 10.27; N, 5.82.

6,6'-Di-(2-methoxycarbonylethylthio)-2,3,4,2',3',4'-hexa-*O*-tetradecyl-α,α'-trehalose (159). To a solution of **77** (120 mg, 0.086 mmol) in dry DMF (5 mL), Cs_2CO_3 (89 mg, 0.27 mmol) and methyl 2-mercaptopropionate (33 μL, 0.27 mmol) were added, under Ar atmosphere, and the reaction mixture was stirred at 70 ºC overnight. The reaction mixture was concentrated and the residue was purified by column chromatography (1:35 → 1:30 → 1:20 EtOAc-cyclohexane). Yield: 150 mg (47%). R_f = 0.17 (1:15 EtOAc-cyclohexane); $[\alpha]_D$ = +78 (*c* 1.0, DCM); IR: ν_{max} = 2922, 2852, 1736, 1097 cm^{-1}.

^{1}H NMR (300 MHz, $CDCl_3$): δ = 5.08 (d, 2 H, $J_{1,2}$ = 3.4 Hz, H-1), 3.99 (ddd, 2 H, $J_{4,5}$ = 9.4 Hz, $J_{5,6b}$ = 6.5 Hz, $J_{5,6a}$ = 2.5 Hz, H-5), 3.82-3.74 (m, 4 H, OCH_2), 3.67-3.43 (m, 10 H, OCH_2, H-3), 3.66 (s, 6 H, OCH_3), 3.20 (dd, 2 H, $J_{2,3}$ = 9.4 Hz, H-2), 3.15 (t, 2 H, $J_{3,4}$ = 9.4 Hz, H-4), 2.79 (t, 4 H, $J_{H,H}$ = 7.4 Hz, SCH_2), 2.75 (dd, 2 H, $J_{6a,6b}$ = 13.5 Hz, H-6a), 2.69 (dd, 2 H, H-6b), 2.59 (t, 4 H, $J_{H,H}$ = 6.4 Hz, CH_2COO), 1.58-1.47 (m, 12 H, CH_2), 1.23 (bs, 120 H, CH_2), 0.86 (t, 18 H, $J_{H,H}$ = 7.4 Hz, CH_3).

^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 172.3, 172.2 (CO), 92.1 (C-1), 80.9 (C-3), 80.5 (C-2), 80.3 (C-4), 73.5, 73.2, 71.6 (OCH_2), 71.2 (C-5), 51.6 (OCH_3), 34.7 (CH_2S), 34.0 (C-6), 31.9 (*C*H_2COO), 30.6, 30.5, 30.2, 29.7, 29.3, 28.4, 27.0, 26.3, 26.2, 22.6 (CH_2), 14.0 (CH_3).

ESIMS: *m/z* = 882.1 $[M + 2Na]^{2+}$, 1747.4 $[M + 2Na]^{+}$. Anal. Calcd for $C_{104}H_{202}O_{13}S_2$: C, 74.42; H, 11.80; S, 3.72. Found: C, 72.58; H, 12.00; N, 3.64.

6,6'-Di-(2-carboxylatoethylthio)-2,3,4,2',3',4'-hexa-*O*-hexyl-α,α'-trehalose disodium salt (160). To a solution of **158** (140 mg, 0.133 mmol) in dry dioxane (12 mL), 1 M NaOH (2 mL) was dropwise added and the reaction mixture was stirred at 40 ºC overnight. The reaction mixture was neutralized with 0.1 N HCl, aqueous solution was extracted with DCM (3 x 10 mL), organic phase was dried ($MgSO_4$), filtered and concentrated. Yield: 110 mg (81%). $[\alpha]_D$ = +113 (*c* 1.0, DCM); IR: ν_{max} = 2926, 2858, 1713, 1094 cm^{-1}.

^{1}H NMR (300 MHz, $CDCl_3$): δ = 5.09 (d, 2 H, $J_{1,2}$ = 3.6 Hz, H-1), 3.96 (ddd, 2 H, $J_{4,5}$ = 9.3 Hz, $J_{5,6b}$ = 6.3 Hz, $J_{5,6a}$ = 2.3 Hz, H-5), 3.86-3.77 (m, 4 H, OCH_2), 3.69-3.46 (m, 10 H, OCH_2, H-3), 3.23 (dd, 2 H, $J_{2,3}$ = 9.3 Hz, H-2), 3.17 (t, 2 H, $J_{3,4}$ = 9.3 Hz, H-4), 2.86 (dd, 2 H, $J_{6a,6b}$ = 14.3 Hz, H-6a), 2.84 (t, 4 H, $J_{H,H}$ = 6.7 Hz, SCH_2), 2.68 (dd, 2 H, H-6b), 2.66 (m, 4 H, CH_2COO), 1.62-1.48 (m, 12 H, CH_2), 1.36-1.25 (m, 36 H, CH_2), 0.90-0.85 (m, 18 H, CH_3).

^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 178.0 (CO), 91.7 (C-1), 81.0 (C-3), 80.5 (C-2), 80.4 (C-4), 73.5, 73.2, 71.7 (OCH_2), 71.4 (C-5), 35.1 (C-6), 34.2 (CH_2S), 31.8 (*C*H_2COO), 31.7, 30.6, 30.4, 30.2, 28.2, 25.9, 25.8, 22.6 (CH_2), 14.4 (CH_3).

ESIMS: *m/z* = 1020.9 $[M - H]^-$. Anal. Calcd for $C_{54}H_{102}O_{13}S_2$: C, 63.37; H, 10.04; S, 6.27. Found: C, 63.69; H, 10.21; N, 5.19.

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